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14. ABSTRACT The objective of the current proposal is to perform immunotherapy to eradicate prostate cancer and at the same time to avoid the development of autoimmune disease. The proposal contains two tasks. The first task is a combination of IL-2 based tumor-reactive T cell adoptive therapy with the TGF-beta based gene therapy for the treatment of mouse prostate cancer. The second task is A tetracycline inducible TGF-beta based gene therapy. At the time of this report, we have completed Task 1 and three papers have been published. (Cancer Research 65:1761-1769, 2005; Prostate 66:235-247, 2006; Molecular Cancer Therapeutics 5:1733-1743, 2006). Currently, we are in the process of to conduct the final phase of the studies described in Task 2.					
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INTRODUCTION:

During the funding period, we have completed studies described in Task 1. Currently, studies described in Task 2 are underway. Briefly, the progress can be summarized by three papers that have been published (see appendix). In these papers, we reported the successful eradication of mouse prostate cancer by adoptive transfer of tumor-reactive TGF-beta insensitive CD8+ T cells in tumor bearing mice.

BODY:

Task 1. To perform a combination of IL-2 based tumor-reactive TGF-beta insensitive CD8+ T cells in an adoptive therapy of tumor bearing mice (see publication by Zhang et al, 2005)

- * We have successfully isolated tumor-reactive CD8+ T cells from donor mice by vaccinating these mice with irradiated TRAMP-C2 mouse prostate cancer cells. The spleen of these vaccinated mice was isolated and CD8+ T cells were isolated.

- * The isolated CD8+ T cells were expanded ex vivo in the presence of lysates of TRAMP-C2 cells, irradiated spleen cells (as the antigen presenting cells), IL-2, and antiCD3 antibody.

- * We have inserted a dominant negative TGF- β type II receptor (dnT β RII) expression vector MSC retrovirus gene into the above mentioned tumor reactive CD8+ T cells. Under a separate start site, a green fluorescent protein expressed vector was inserted into the same retrovirus gene.

- * We have successfully transferred the tumor-reactive TGF-beta insensitive CD8+ T cells to the tumor-bearing male C57BL/6 mice.

- * Tumor cells were injected into above recipient mice at 21 days prior to adoptive transfer of CD8+ T cells. At 40 days after the adoptive transfer, animals were sacrificed for inspection of the status of metastasis of TRAMP-C2 mouse prostate cancer cells into the lung.

- * In mice received naïve CD8+ T cells, the majority of the tumors were not rejected. In animals received tumor-reactive control CD8+ T cells, there was a partial tumor rejection. However, in animals received adoptive transfer of tumor-reactive TGF-beta insensitive CD8+ T cells, most of the tumors were rejected with no apparent toxicity.

- * Since tumors were eradicated without the use of IL-2, the combination of IL-2 and the adoptive transfer experiment was deemed unnecessary and we did not pursue this approach further.

Task 2: A tetracycline inducible TGF-beta based gene therapy.

In the original application, we proposed to use the tetracycline-inducible system to express the TBRIIDN gene in immune cells. This task will be carried out jointly by Dr. Lee and Victoria Liu (a graduate assistant). We found out that such a tetracycline-inducible system was not suitable for a stable transfection for the vector to be permanently integrated into the chromosome of the target cells. We have decided to change into a thymidine kinase inducible system, as described below.

In 1989, the team led by Dr. Ron Evans has developed a new transgenic mouse system in which ablation of a specific cell type is thymidine kinase

dependent (Borrelli et al, 1989; Heyman et al, 1989). In such a system, inserted herpes simplex virus thymidine kinase (*HSV-tk*) gene products can phosphorylate certain nucleotide analogues such as ganciclovir (GCV) that are not metabolized by conventional cellular enzymes. Phosphorylated nucleoside analogues such as GCV triphosphate are potent toxic metabolites for cells. Nevertheless, neither GCV nor HSV-tk alone is harmful to cells. Hence, this conditional cell-depleting effect is achieved by expressing *HSV-tk* with a cell-specific promoter. Such a HSV-tk/GCV mediated conditional cell-depleting system has been used for the depletion of specially selected cell types by many investigators (Rettig et al, 2004). In the present study, we proposed to use this system to conditionally deplete the TGF- β insensitive bone marrow cells in recipient hosts (mice and humans) when it is necessary to eliminate the transferred TGF- β insensitive CD8+ T cells.

Construction and testing of the T β RIIDN-tk vector:

In collaboration with Dr. Isaac Kim, currently at the Cancer Institute of New Jersey, we have modified the original retroviral vector with T β RIIDN (Shah et al, 2002a,b) to connect the HSV-tk as the intracellular domain of the T β RIIDN molecule. As such, the viral vector is still function as the dominant negative T β RII. When this viral vector is infected into the target cell, the cell will not only be insensitive to TGF- β but will also express thymidine kinase as depicted below.

T β RIIDN-tk Construct:

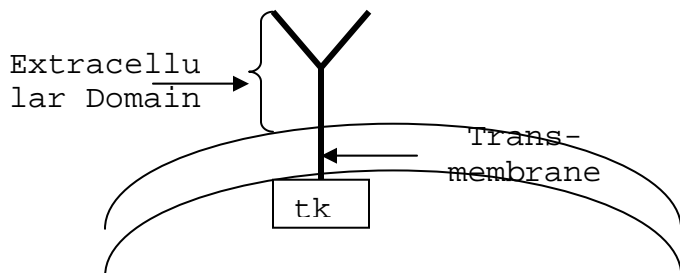
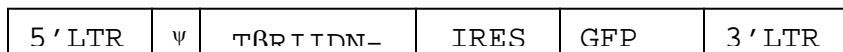


Fig 1

The control vector to the above construct is designed by excising the extracellular domain of the T β RIIDN molecule but retaining the transmembrane domain so that the HSV-tk is still anchored as part of the intracellular domain of the truncated T β RIIDN. When this control vector is infected into the target cell, the cell is unable to bind TGF- β but will express thymidine kinase as indicated below. (see next page for Fig 2)

TRANS-tk Construct (Control vector):

5' LTR	Ψ	TRANS-tk	IRES	GFP	3' LTR
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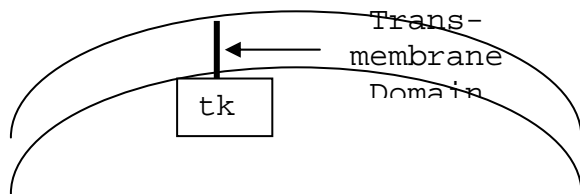
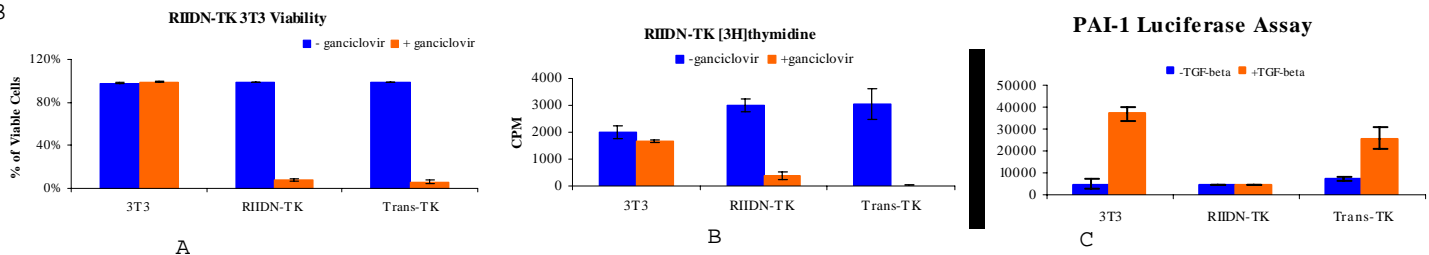


Fig 2

Results of our preliminary study using NIH 3T3 cells as the target have demonstrated efficacy of these constructs. The infection rate in NIH-3T3 cells was over 90% and the sensitivity to TGF- β in these cells is depicted below. When these cells were treated with GCV (10 ug/ml), they rapidly lose the viability and the rate of ^3H -thymidine incorporation. They also have lost the sensitivity to TGF- β , as indicated by a lack of PAI-I luciferase activity when they were treated with TGF- β 1.

Fig 3



In the last study, we will conduct in vivo anti-tumor experiment using the retroviral vector with T β RIIDN-HSV-tk as the vector to infect the bone marrow cells in tumor bearing mice as described earlier (Shah et al, 2002a; Zhang et al, 2005).

KEY RESEARCH ACCOMPLISHMENTS:

- We have developed an immunotherapy approach in which we perform adoptive transfer of tumor-reactive TGF-beta insensitive CD8+ T cells into tumor bearing mice. These animals were able to reject established mouse prostate tumors metastasized into the lung.

REPORTABLE OUTCOMES:

As a result of this research funded by the Department of Defense, we have completed a manuscript. (see appendix).

CONCLUSIONS:

Immunotherapy using adoptive transfer of immune cells is a promising approach for treating cancer patients. The presence of tumor infiltrating lymphocytes (TIL) in the tumor parenchyma has been recognized for three decades (Ioachim, 1979). TIL were isolated from surgical specimens, clonally expanded by ex vivo culture, and adoptively transferred to the cancer patients with variable results (Economou, J.S. et al. 1996; Figlin, R.A. et al. 1999). Recently, Yee and coworkers selected antigen specific

CD8⁺ T cells for ex vivo expansion and transferred these cells into patients. These CD8⁺ T cells did not persist, requiring repeated transfer of CD8⁺ T cells in order to elicit responses from the patients (Yee, C. et al, 2002). Rosenberg and colleagues treated autologous TIL cells with IL-2 for ex vivo expansion and then transferred them to patients. Again, in order for these cells to "engraft", lymphodepletion was necessary (Rosenberg and Dudley, 2004). These results, although impressive, fall short of our expectation, i.e., total elimination of tumor cells in most patients.

One of the reasons for the failure of TIL in treating cancer is perhaps the fact that the functional role of TIL in cancer has been a subject of controversy (Miesche et al, 1989). Initially, it appeared that the presence of TIL in the tumor could correlate to prognosis (Svennevig et al, 1984). Subsequent studies showed that TIL were functionally impaired (Whiteside, 1992; Meischer. et al, 1998; Reichert, T.E. et al, 2002). This impairment of TIL has been attributed to effects exerted by the tumor microenvironment (Rabinowich, H. et al, 1996). When developing an immune-based strategy for cancer therapy, in addition to immune stimulation, the issue of overcoming tumor-derived immune suppression must be taken into consideration (Dudley and Rosenberg, 2003). There are 6 potential mechanisms of tumor immune escape: loss of tumor antigen expression; variations in tumor antigen; defects in the transporter associated with antigen presentation; defects in expression of the MHC heavy chain; expression of immunoprotective molecules and release of molecules by tumor cells that disrupt T-cell signaling or induce T-cell death; and upregulation of the expression of immunoprotective molecules (PI9, FLIP and the IAP family) that counteract the effects of FASL, granzyme B, CXCL12, FLIP, FLICE, IAP, IL-10, PI9, RCAS1 and TGF- β . Among these molecules, TGF- β is an important immunosuppressant (Yee and Greenberg, 2002).

TGF- β has been recognized as a potent immunosuppressive factor (Wojtowicz, 1997; Letterio and Roberts, 1998; Fortunel et al, 2000; Gorelik and Flavell, 2001; Kao. et al, 2003). The high levels of TGF- β produced by cancer cells have a negative effect on surrounding cells, including the host immune cells and have been implicated to play a role in tumor escape from immune surveillance (Won, J. et al, 1999; de Visser and Kast, 1999). Besides the tumor, the immune system, in response to the presence of tumor, is also able to produce a significant amount of TGF- β to down regulate immune surveillance (Terabe, M. et al, 2003).

In light of the above discussion, TGF- β appears to be an attractive target for anti-cancer therapy. Attempts to take advantage of the properties of TGF- β for the treatment of cancer have been reported. Gorelik and Flavell (2001) first described the immune-mediated eradication of tumors through the blockade of TGF- β signaling in T cells. These investigators used transgenic mice with TGF- β null expression targeted specifically to T cells. Subsequently, our study using transplant of TGF- β -insensitive bone marrow cells also demonstrated a total rejection of metastatic tumor cells (Shah et al, 2002a,b). However, in both studies, due to the non-specific nature of the immune cells, autoimmune disease eventually developed in the hosts. Results of our recent study have shown that adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells were able to eradicate established lung metastasis of mouse prostate

cancer cells, TRAMP-C2 (Zhang et al, 2005, 2006a,b). In the present study, we continue to employ this novel gene therapy approach to investigate the ability these CD8⁺ T cells to infiltration into the tumor parenchyma. Here, we report that these CD8⁺ T cells show a distinct ability to infiltrate into established tumors, secrete relevant cytokines, and induce apoptosis of tumor cells.

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APPENDICES:

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Adoptive Transfer of Tumor-Reactive Transforming Growth Factor- β -Insensitive CD8⁺ T Cells: Eradication of Autologous Mouse Prostate Cancer

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Abstract

Transforming growth factor (TGF)- β is a potent immunosuppressant. Overproduction of TGF- β by tumor cells may lead to tumor evasion from the host immune surveillance and tumor progression. The present study was conducted to develop a treatment strategy through adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells. The mouse TRAMP-C2 prostate cancer cells produced large amounts of TGF- β 1 and were used as an experimental model. C57BL/6 mice were primed with irradiated TRAMP-C2 cells. CD8⁺ T cells were isolated from the spleen of primed animals, were expanded *ex vivo*, and were rendered TGF- β insensitive by infecting with a retrovirus containing dominant-negative TGF- β type II receptor. Results of *in vitro* cytotoxic assay revealed that these CD8⁺ T cells showed a specific and robust tumor-killing activity against TRAMP-C2 cells but were ineffective against an irrelevant tumor line, B16-F10. To determine the *in vivo* antitumor activity, recipient mice were challenged with a single injection of TRAMP-C2 cells for a period up to 21 days before adoptive transfer of CD8⁺ T cells was done. Pulmonary metastasis was either eliminated or significantly reduced in the group receiving adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells. Results of immunofluorescent studies showed that only tumor-reactive TGF- β -insensitive CD8⁺ T cells were able to infiltrate into the tumor and mediate apoptosis in tumor cells. Furthermore, transferred tumor-reactive TGF- β -insensitive CD8⁺ T cells were able to persist in tumor-bearing hosts but declined in tumor-free animals. These results suggest that adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells may warrant consideration for cancer therapy. (Cancer Res 2005; 65(5): 1761-9)

Introduction

Adoptive therapy using antigen-specific immune cells from patients has become an attractive approach for tumor immunotherapy (1). The procedure has been effective in cases of low tumor burden. Increased knowledge of immunoregulation and immune function of effector cells has led to the development of specific cell therapy with the objective of targeting tumor cells for destruction.

The ultimate effector cell that facilitates tumor rejection in preclinical animal models is CD8⁺ T cells (1).

Historically, adoptive transfer of lymphokine-activated killer cells was first attempted, in which *ex vivo* culture of autologous lymphocytes with interleukin 2 (IL-2) to increase the number of activated effector cells. However, a clinical benefit was not shown by this approach (2). Subsequently, tumor-infiltrating lymphocytes were isolated from surgical specimens, clonally expanded by *ex vivo* culture with IL-2, and adoptively transferred to the patient (3). Although the initial results were promising, results of a phase III randomized trial using CD8⁺ tumor-infiltrating lymphocytes in combination with IL-2 failed to show an improved response in patients with metastatic renal cell carcinoma (4). Two recent studies represent the state-of-the-art strategies in adoptive therapy for cancer. In the first study (5), authors selected antigen-specific CD8⁺ T cells for *ex vivo* expansion and transfer into patients. Responses were remarkable but these CD8⁺ T cells did not persist, requiring repeated transfer of CD8⁺ T cells to elicit responses from the patients. In the second study (6), authors treated autologous tumor-infiltrating lymphocyte cells with IL-2 for *ex vivo* expansion and then transferred to patients following lymphodepletion. Although long-term engraftment was achieved, only 4 of 35 patients showed complete response. These results, although impressive, fall short of our expectation (i.e., total elimination of tumor cells in most patients).

In considering tumor immunotherapy, the issue of tumor-derived immune suppression must be taken into consideration (1). It seems that despite the ability to generate immune cells reactive against cancer antigens, tumor escape mechanisms can overpower these immune reactions with an eventual tumor progression (7). Tumor cells have acquired many mechanisms to evade the host immune surveillance (8, 9). One of such possibilities has been the down-regulation of tumor antigen processing (10). Tumor-specific CD4⁺CD25⁺ T regulatory cells can also inhibit CD8⁺ T-cell function (11). Tumor-derived immunosuppressive cytokines, including vascular endothelial growth factor, IL-10, and transforming growth factor (TGF)- β (9, 12–14), also contribute to tumor evasion of the host immune surveillance. In the present study, we propose to focus on TGF- β -mediated evasion of immune surveillance.

Tumor cells secrete large amounts of TGF- β . High levels of TGF- β produced by cancer cells have a negative impact on surrounding cells, including the host immune cells (15). TGF- β is a potent tumor-induced immunosuppressant (8, 12, 16–20). Therefore, TGF- β seems to be an attractive target for anticancer therapy. The first piece of work describing immune-mediated eradication of tumors through the blockade of TGF- β signaling in T cells was reported by Gorelik and Flavell (17). These authors used transgenic mice with

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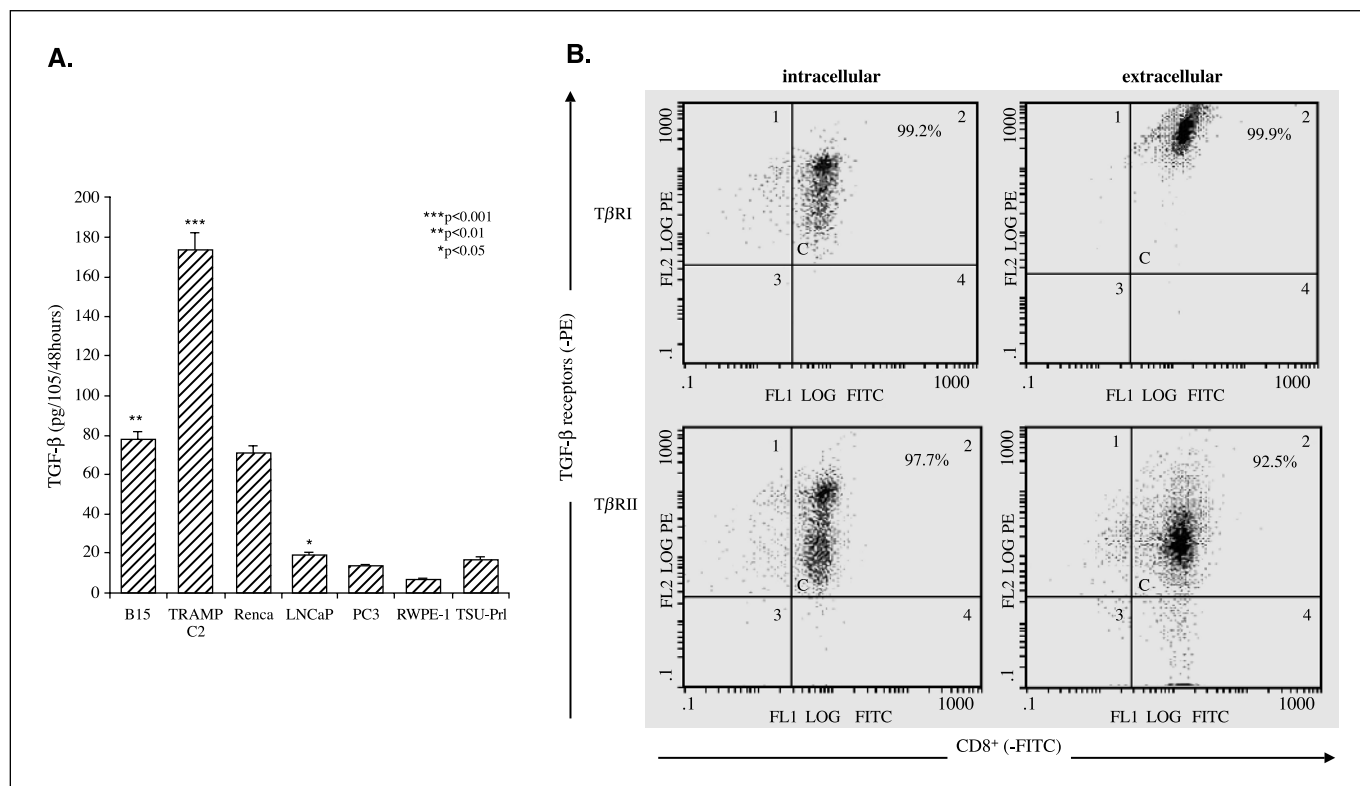


Figure 1. A, secretion of TGF- β 1 levels by different cell lines. TGF- β 1 present in the conditioned media over a period of 48 hours was determined by ELISA (see Materials and Methods) and was expressed as pg per 10^5 cells per 48 hours. TRAMP-C2, mouse prostate cancer cell line; B16, mouse melanoma cell line; Renca, mouse renal cell carcinoma cell line; LNCaP and PC3, human prostate cancer cell lines; TSU-Pr1, human bladder cancer cell line; RWPE-1, immortalized human normal prostate epithelium cell line. Bars, SD. B, expression of TGF- β type I (*T β RI*) and type II receptors (*T β RII*) in normal mouse splenic CD8 $^+$ T cells. Analysis was done by double-labeled immunofluorescent FACS (CD8 $^+$ -FITC, TGF- β receptors-phycoerythrin; see Materials and Methods).

TGF- β null expression targeted specifically to T cells. Subsequently, our study using transplant of TGF- β -insensitive bone marrow cells also showed a total rejection of metastatic tumor cells (19, 20). However, in both studies, due to the nonspecific nature of the immune cells, autoimmune disease eventually developed in the hosts.

In the present study, we attempted to combine the above two advanced technologies by employing adoptive transfer of tumor-reactive TGF- β -insensitive CD8 $^+$ T cells into tumor-bearing mice. Here, we report that these CD8 $^+$ T cells showed a robust antitumor activity with little or no apparent toxicity.

Materials and Methods

Mice and Cells. Male C57BL/6 mice ages 6 to 8 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University's Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Northwestern University. TRAMP-C2 cells obtained from Dr. N. Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA) were maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 100 units/mL penicillin, and 100 μ g/mL streptomycin.

Generation of Tumor-Reactive CD8 $^+$ T Cells. Mice were primed with irradiated TRAMP-C2 cells (5×10^6 per mice at 20,000 Ci) by s.c. injection every 10 days for a total of three inoculations. Two weeks following the last vaccination, splenic CD8 $^+$ T cells were isolated by using murine T cell CD8 $^+$ subset column kit (R&D Systems, Minneapolis, MN) and were

expanded (10^5 /mL) in the presence of TRAMP-C2 lysates (1×10^6) and irradiated autologous splenocytes (1×10^6 /mL at 3,000 Ci) in medium containing RPMI 1640 with 10% fetal bovine serum, IL-2 (50 units/mL), anti-CD3 $^+$ monoclonal antibody (30 ng/mL, R&D Systems), HEPE (25 mmol/L), L-glutamine (4 mmol/L), and 2-ME (25 mmol/L). Media were changed every 3 days.

Infection with Retrovirus Containing Dominant Negative TGF- β Type II Receptor or Green Fluorescent Protein. CD8 $^+$ T cells were cultured for at least 10 weeks before they were infected with the murine stem cell virus retrovirus containing the dominant-negative TGF- β type II receptor (T β RIIDN) and green fluorescent protein (GFP; Fig. 2A; refs. 19, 20). The infection efficiency was 93.9% for the T β RIIDN vector and 92.8% for the GFP control vector (Fig. 2B). There were three types of CD8 $^+$ T cells. The first type was tumor-reactive TGF- β -insensitive CD8 $^+$ T cells (tumor-reactive CD8 $^+$ T cells infected with the virus containing T β RIIDN). The second type was tumor-reactive CD8 $^+$ T cells infected with the virus containing the GFP control vector. The third type was naive CD8 $^+$ T cells, which were freshly isolated from the spleen of naive donor animals without any treatment.

In vitro CTL Assay. The above three types of CD8 $^+$ T cells were subjected to a standard 51 Cr release assay (20). TRAMP-C2 cells were used as targets. An irrelevant cancer cell line, mouse melanoma cell line, B16-F10, was used as a nonspecific control. Target cells were labeled with 0.1 mCi of 51 Cr per 10^6 cells for 4 hours at 37°C, followed by 5 washes in HS-4 media and were seeded in 96-well U-bottomed plates (5,000 cells/well). CD8 $^+$ T cells were added at different effector/target ratios (1:1 to 100:1) for 5 hours. The supernatants were harvested using the Skatron filters (Skatron Instruments, Sterling, VA) and the released radioactivity was measured using a gamma counter (LKB Wallac, Turku, Finland). The percent of specific lysis was determined as $100 \times ([\text{Experimental } ^{51}\text{Cr Release} - \text{Spontaneous } ^{51}\text{Cr Release}] / [\text{Maximum } ^{51}\text{Cr Release} - \text{Spontaneous } ^{51}\text{Cr Release}])$.

Release)). The maximum release was determined by adding 2% SDS to target cells.

In vivo Antitumor Assay. Mice received a single injection of 5×10^5 TRAMP-C2 cells via the tail vein. Adoptive transfer of CD8⁺ T cells (2×10^6) was done on either day 3, 7, or 21 following tumor cell injection. Mice were maintained on antibiotics (sulfamethoxazole-trimethoprim) for a minimum of 2 weeks to prevent opportunistic infection after the injection. Forty days after the adoptive transfer, all animals were sacrificed. Some animals were sacrificed sooner than 40 days due to poor health conditions. Serum levels of IFN- γ and IL-2 were determined by ELISA. Splenic CD8⁺ T cells were

isolated, and the percentage of GFP-positive CD8⁺ T cells in each spleen was calculated following analysis by flow cytometry.

Histologic Procedures. For each animal, upon euthanasia, the lung was excised, fixed in formalin, embedded in paraffin, and serially sectioned at 4- μ m thickness until the block was exhausted. Routine H&E staining was done at an interval of every 10 sections. The unstained paraffin sections were used for studies described below.

Nuclear Staining, Apoptosis Assay, and CD8⁺ Staining. Tissue sections were subjected to apoptosis assay by using the TUNEL apoptosis kit (Upstate, Lake Placid, NY) and were labeled with Avidin-FITC

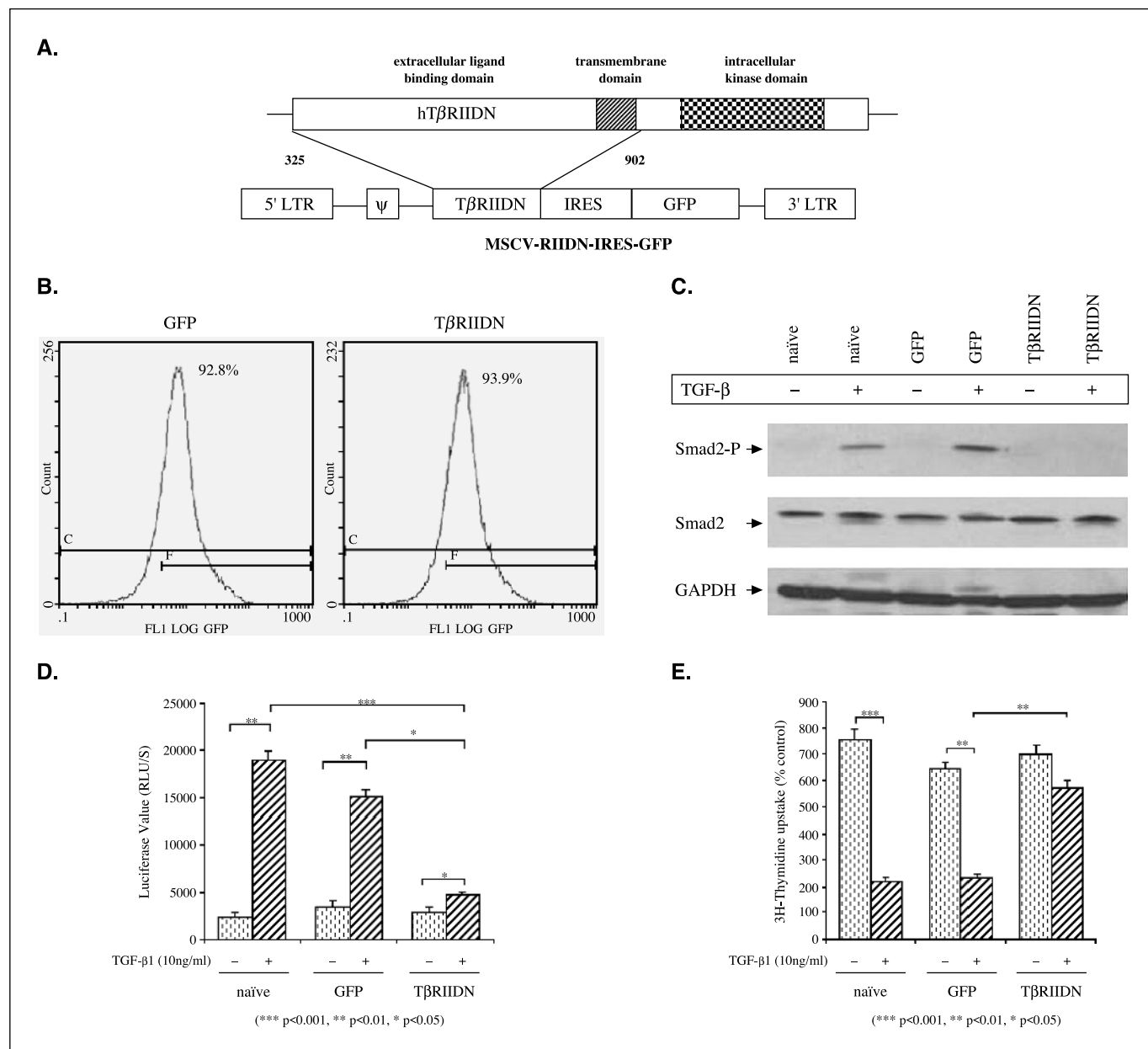


Figure 2. Structure, function, and expression of dominant-negative T β RIIDN. **A**, schematic diagram of the murine stem cell virus (MSCV) retroviral construct. A truncated sequence of the human T β RIIDN, lacking the intracellular kinase signaling domain, was cloned into the pMig-internal ribosomal entry sequence (IRES)-GFP vector. The control construct (not shown) contained the GFP vector only and without the T β RIIDN sequence (325-902 bp). **B**, fluorescent-activated cell sorting analysis of murine CD8⁺ T cells transfected with the T β RIIDN vector (93.9%) and the GFP control vector (92.8%). The high efficiency of infection of the viral transgene into CD8⁺ T cells allowed us to perform adoptive transfer directly without the need of sorting. **C-E**, series of functional analysis done following the treatment of CD8⁺ T cells with 10 ng/mL TGF- β 1. **C**, phosphorylation of Smad-2 observed in naïve CD8⁺ T cells and in tumor-reactive CD8⁺ T cells infected with the GFP control vector but not observed in tumor-reactive CD8⁺ T cells infected with the T β RIIDN vector. Blots were stripped and reprobed with anti-Smad-2 and anti-GAPDH antibodies as loading controls. **D**, luciferase activity in CD8⁺ T cells transfected by TGF- β signal-specific plasminogen activator inhibitor-1 promoter-luciferase reporter construct and treated with TGF- β 1. **E**, [³H]-thymidine incorporation into CD8⁺ T cells before and after the treatment with TGF- β 1. Bars, SD.

(green, 50 μ L). The same slides were treated with blocking buffer and probed for CD8 with rat monoclonal antibody, which was labeled with APC (red; 2 μ g/mL, Santa Cruz Biotechnology, Santa Cruz, CA). Finally, the same slides were stained for cell nuclei with VECTASHIELD mounting media (blue; Vector Laboratories, Burlingame, CA). They were viewed with Nikon TE2000-U fluorescent microscopy (Nikon Co., Tokyo, Japan). Images were digitized by Photoshop 7.0 software.

Expression of TGF- β Receptors in CD8⁺ T Cells. Normal CD8⁺ T cells, isolated from freshly harvested spleens, were subjected to fixation and permeabilization in preparation for immunofluorescent staining and flow cytometry analysis. Cells were stained with phycoerythrin-conjugated monoclonal antibody against TGF- β type I receptor (1:100 dilution; Santa Cruz Biotechnology), and TGF- β type II receptor (1:50 dilution, Santa Cruz Biotechnology). They were then stained with FITC-conjugated monoclonal antibody against CD8 (Santa Cruz Biotechnology). These cells were subjected to dual analysis for phycoerythrin and FITC by flow cytometry.

Western Blot Analysis for SMAD-2 Phosphorylation. CD8⁺ T cells were treated with or without 10 ng/mL of TGF- β 1 for 16 hours (19). Cell

lysates were prepared by adding radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, 1% NP40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, and 1 mmol/L NaF) to cell pellets. Approximately 30 μ g of total protein extract were loaded onto 10%

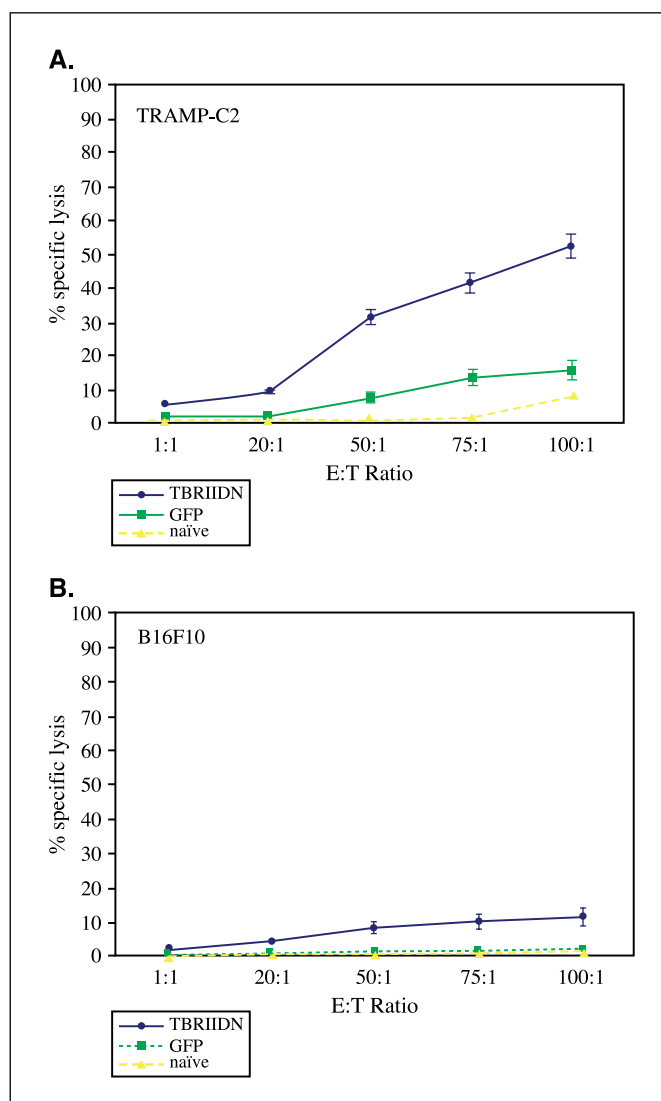


Figure 3. *In vitro* CTL assays. CTL was done using the conventional ⁵¹Cr release assay (see Materials and Methods). Naïve CD8⁺ T cells, GFP, and T β RIIDN transfected CD8⁺ T cells were cocultured with ⁵¹Cr-labeled targets at the specified E/T ratios. **A**, TRAMP-C2 mouse prostate cancer cells were used as the targets; **B**, B16-F10 mouse melanoma cells were used as targets. Point, average observations obtained from eight wells; bars, SD.

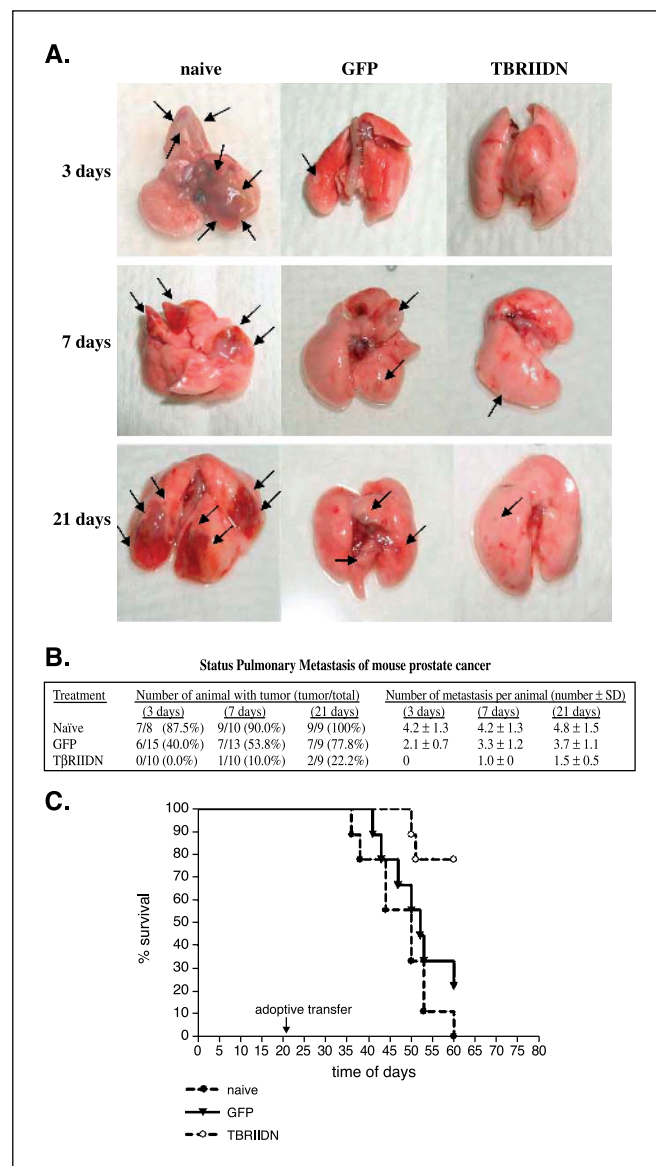


Figure 4. Status of pulmonary metastasis of TRAMP-C2 tumors in mice received adoptive transfer of three types of CD8⁺ T cells. Recipient mice received a single injection of TRAMP-C2 cells (5×10^5). At 3, 7, or 21 days following the initial tumor challenge, adoptive transfer of CD8⁺ T cells was done. Animals were sacrificed at 40 days following the adoptive transfer or sooner due to poor health conditions. **A**, representative gross feature of lung tissues from tumor-bearing mice at 40 days following the administration of adoptive transfer. Arrows, metastatic sites. **B**, status of pulmonary metastasis of mouse prostate cancer. The presence of gross and microscopic pulmonary metastasis in each treatment group was tabulated and expressed as the number of animals with pulmonary metastasis (% total animals in each group) as well as the average number of metastatic sites per animal. Results of the statistical analysis, using the χ^2 test, indicated that values in the T β RIIDN group are significantly different from those of other two groups ($P < 0.001$). Also, values in the GFP group are significantly different from those of the naïve group ($P < 0.001$). **C**, Kaplan-Meier survival curve of tumor-bearing mice received adoptive transfer of naïve CD8⁺ T cells (dotted line with solid circles), GFP control vector (solid line with solid circles), and T β RIIDN-transfected CD8⁺ T cells (dotted line with open circles). $P < 0.05$ according to the log-rank test for the T β RIIDN group versus the naïve or GFP group.

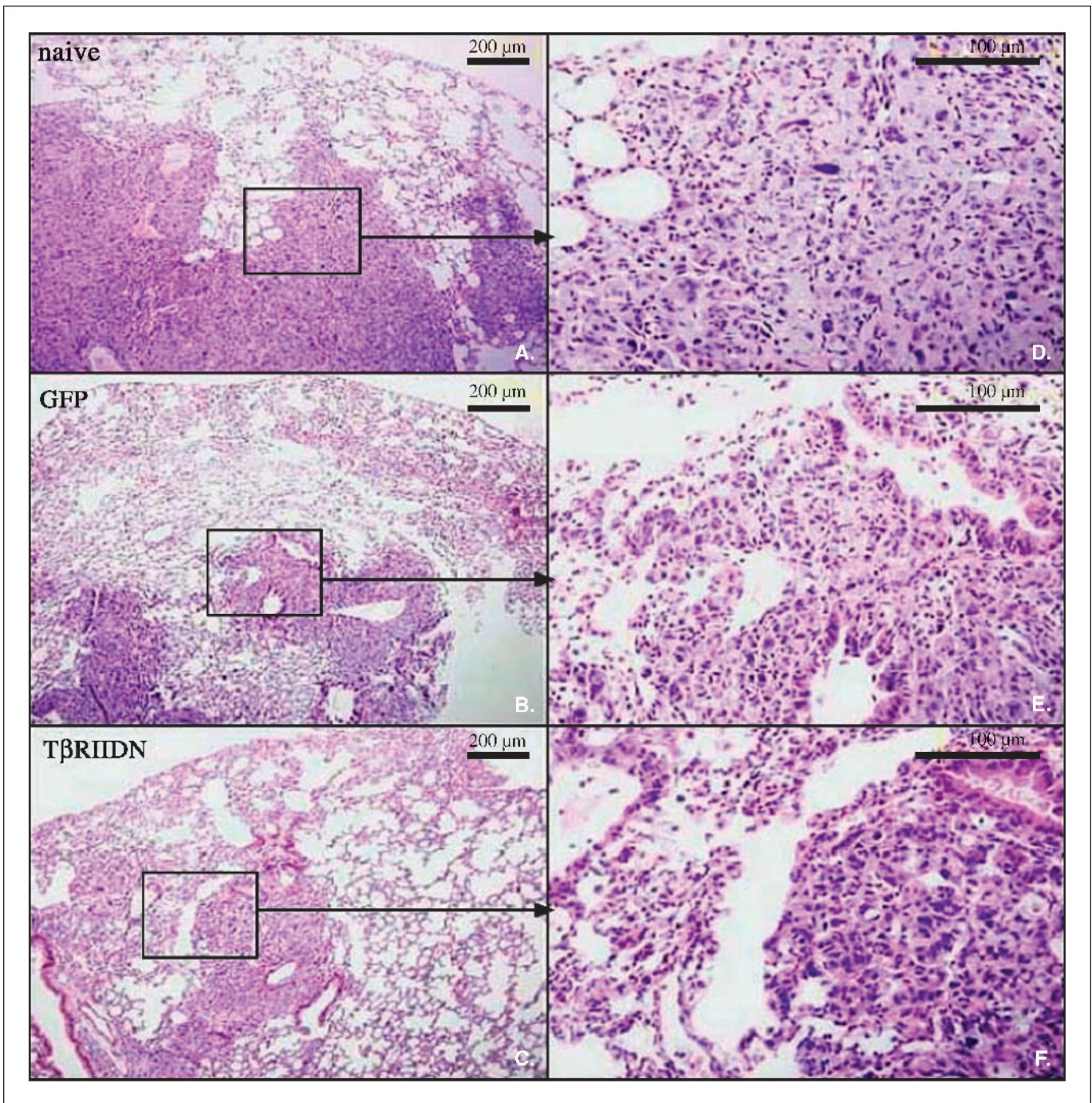


Figure 5. Representative histologic features (H&E staining) of metastatic tumor nodules in the lungs from animals received adoptive transfer of naive CD8⁺ T cells (A and D), GFP-infected tumor reactive CD8⁺ T cells (B and E), and T β RIIDN-infected tumor reactive CD8⁺ T cells (C and F) mice 40 days following the adoptive transfer. These animals received injection of tumor cells 7 days before the adoptive transfer. A, lung tissue of a mouse which received adoptive transfer of naive CD8⁺ T cells showing a portion of a large tumor (4 mm in diameter) with marked cytologic polymorphism (D). B, lung tissue of a mouse which received adoptive transfer of tumor-reactive control CD8⁺ T cells, which contained the control GFP vector. There are two smaller tumor nodules (0.5 and 0.6 mm in diameter, respectively), which showed some immune cell infiltration and degenerative changes of tumor cells (E). C, lung tissue of a mouse which received adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells. There is a smaller tumor nodule (0.5 mm in diameter). Within this tumor, heavy immune cell infiltrates and marked degenerative changes of tumor cells can be seen (F). This impression was confirmed in Fig. 6A.

acrylamide gel in Tris-HCl (Bio-Rad, Hercules, CA). Electrophoresis was carried out in Tris-glycine-SDS running buffer and transferred to a polyvinylidene difluoride membrane. Blots were probed for phosphorylated *SMAD-2* with a monoclonal antibody. They were then stripped and reprobed for *SMAD-2* and glyceraldehyde-3-phosphate dehydrogenase. Proteins of interest were detected with the enhanced chemiluminescence

detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom) followed by exposure to Kodak X-OMAT AR film.

Plasminogen Activator Inhibitor-1 Promoter-Reporter Activity Assay. CD8⁺ T cells were transiently transfected with a promoter construct, 3TP-Lux, which contains multiple copies of TGF- β response element, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Cells were treated with

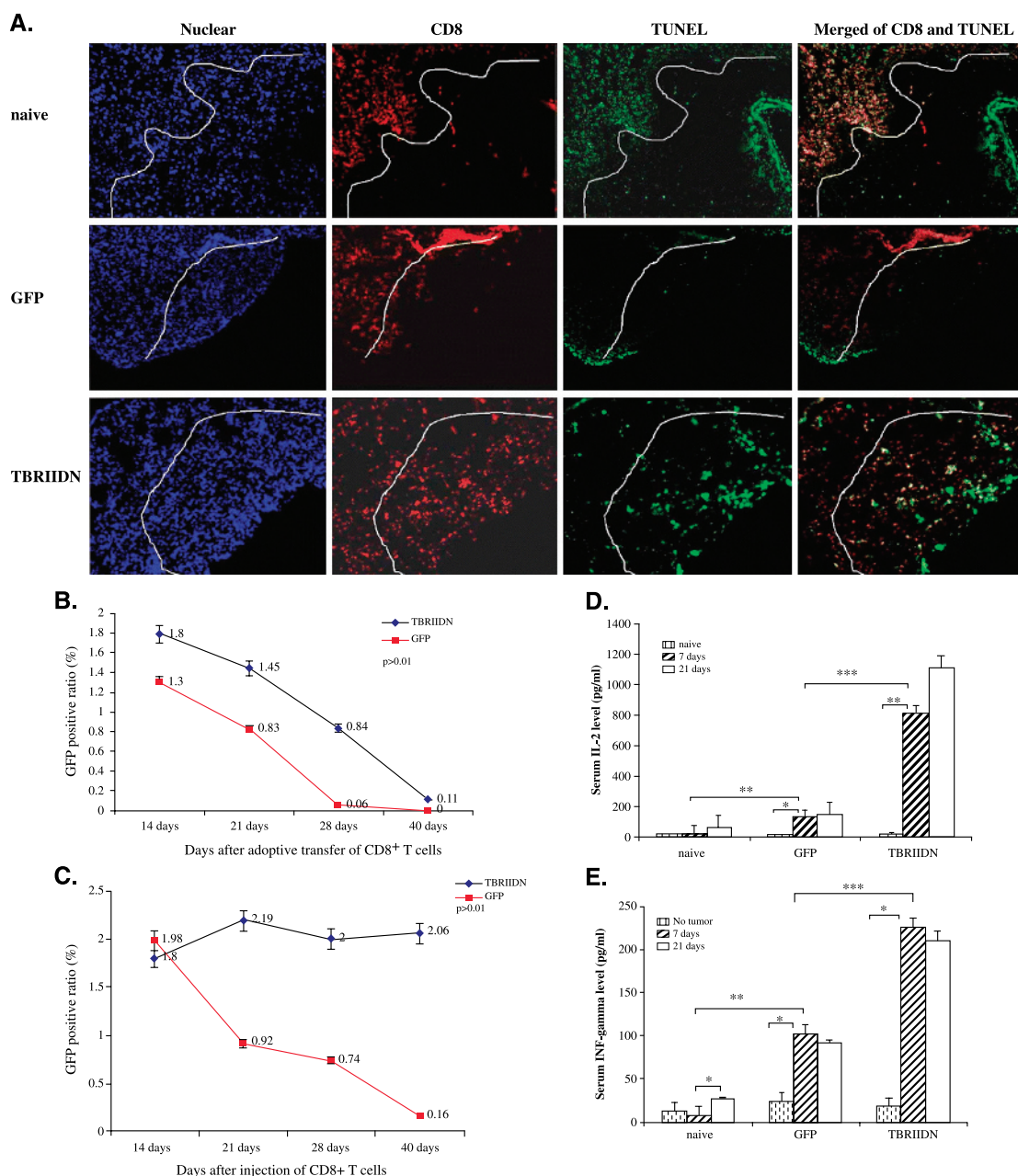


Figure 6. A, immunofluorescent staining for nuclei, CD8⁺ T cells, and apoptosis in pulmonary metastasis. Representative tissue sections of pulmonary metastasis from the day 7 group were simultaneously stained for cell nucleus (blue), CD8⁺ T cells (red), and apoptosis (green). Metastatic sites were identified by the nuclear staining (blue). CD8⁺ T cells (red) were identified mainly in the parenchyma of lung tissues not in the tumor with the exception of the TβRIIDN group, in which CD8⁺ T cells (red) were also found within the tumor lesion. Frequent tumor apoptotic sites (green) were only found in the TβRIIDN group. Although few CD8⁺ T cells were found undergoing apoptosis (yellow), the majority of the apoptotic cells were derived from the tumor cells (green). Magnification, ×40. The fate of adoptively transferred CD8⁺ T cells in recipients of different treatment groups. A total of 2×10^6 CD8⁺ T cells were injected via the tail vein into all recipient mice. At designated time intervals, CD8⁺ T cells from the spleen of each animal were isolated. % GFP-positive CD8⁺ T cells was calculated by fluorescent-activated cell sorting. B, % GFP-positive CD8⁺ T cells in the spleen of tumor-free animals. C, % GFP-positive CD8⁺ T cells in the spleen of tumor-bearing animals. A total of 2×10^6 CD8⁺ T cells were tail vein injected into tumor-bearing C57BL/6 mice. % GFP-positive CD8⁺ T cells in the spleen was analyzed by fluorescent-activated cell sorting. Bars, SD. D, circulating levels of IL-2 and IFN-γ in experimental mice. Serum specimens were collected at the time of animals' euthanasia, which is 40 days following the adoptive transfer of CD8⁺ T cells. Serum level of IL-2 in different groups of mice. E, Serum level of IFN-γ in different groups of mice. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Bars, SD.

10 ng/mL of TGF-β1 for 16 hours. Luciferase activity was assayed by using an assay kit (Promega, San Diego, CA). Activity was normalized based on β-galactosidase expression with pSVβ-galactosidase.

Thymidine Incorporation Assay. CD8⁺ T cells (3×10^4 cells per 24 wells) were treated with or without TGF-β1 (10 ng/mL) for 16 hours.

A medium containing [³H]-thymidine (0.5 μCi/mL; Amersham Biosciences) was introduced and cells were cultured for additional 5 hours. The experiment was terminated by washing with warm serum-free medium. NaOH (0.1 mol/L) was added to all wells (1 mL). An aliquot of 100 μL was removed for measurement of the protein content and the remainder

was used for determining the radioactivity. Thymidine incorporation was expressed as the fraction of counts found in controls.

TGF- β 1 ELISA Assay. TRAMP-C2 cells (1.0×10^7 per T75 flask) were cultured in serum-free media for 24 hours. The medium was replaced for 24 hours. The pooled conditioned medium was collected and concentrated by using YM-3 Centriprep Centrifugal Filter Devices (Millipore Co., Bedford, MA). After activation of TGF- β 1 by treatment with 1 N HCl (0.1 mL per 0.5 mL per conditioned media), the mixture was neutralized by 0.1 mL 1.2 N NaOH/0.5 mol/L HEPES. The ELISA assay was carried out using the Quantikine Human TGF- β 1 Immunoassay Kit from R&D Systems (Minneapolis, MN). The total number of cells in each flask was counted using a Coulter Counter and levels of TGF- β 1 were reported as pg per 10^5 cells per 48 hours.

Fate of Tumor-Reactive CD8 $^+$ T Cells in the Spleen. Because tumor-reactive CD8 $^+$ T cells are labeled with GFP, the percent of GFP positive CD8 $^+$ T cells in the spleen was determined by flow cytometry. This experiment was carried out in both tumor-free animals and in tumor-bearing animals at different time points.

Statistical Methods. All *in vitro* experiments were done in triplicate. Numerical data were expressed as mean \pm SD. ANOVA and multiple range test were done to determine differences of means among different treatment groups. $P < 0.05$ was considered statistically significant. The SPSS 10.0.7 software package (SPSS, Inc., Chicago, IL) was used for analysis. Kaplan-Meier survival curve was analyzed by the log-rank test using the Graphpad Prism 4.02 software (Graphpad Software, Inc., San Diego, CA).

Results

TGF- β 1 Production in TRAMP-C2 Cells. TRAMP-C2 cells secreted 170 pg TGF- β 1 in 10^5 cells over 48 hours. For comparison, TGF- β 1 production was measured in other murine cell lines (B16, Renca), human cancer cell lines (LNCaP, PC3, and TSU-Pr1) and a benign human prostate epithelial line, RWPE-1 (Fig. 1A).

Status of TGF- β Signaling in CD8 $^+$ T Cells. Under normal conditions, CD8 $^+$ T cells expressed high levels of type I and type II TGF- β receptors (Fig. 1B) and they are highly sensitive to the inhibitory effect of TGF- β (12). When CD8 $^+$ T cells were infected with the retrovirus containing T β RIIDN, they became insensitive to TGF- β , as shown by following tests. T β RIIDN infected CD8 $^+$ T cells were insensitive to TGF- β 1 by a lack of SMAD-2 phosphorylation, which was observed in naive CD8 $^+$ T cells or cells infected with GFP control vector (Fig. 2C). Similarly, there was a significant inhibition of plasminogen activator inhibitor-1 promoter-luciferase reporter activity in TGF- β -insensitive CD8 $^+$ T cells in response to TGF- β 1 (Fig. 2D). Finally, these cells were insensitive to TGF- β 1-mediated inhibition of thymidine incorporation assay (Fig. 2E).

***In vitro* Antitumor Activity of Tumor-Reactive TGF- β -Insensitive CD8 $^+$ T Cells.** Tumor-reactive TGF- β -insensitive CD8 $^+$ T cells showed a potent-specific lysis against TRAMP-C2 cells (Fig. 3A). These cells showed a 5-fold more tumor-killing activity than that of TGF- β -sensitive counterpart and 25-fold over that of naive CD8 $^+$ T cells. Both the TGF- β -sensitive and TGF- β -insensitive tumor-reactive CD8 $^+$ T cells showed a reduced tumor-killing activity when incubated with an irrelevant cell line, mouse B16-F10 melanoma cells (Fig. 3B).

***In vivo* Antitumor Activity of Tumor-Reactive TGF- β -Insensitive CD8 $^+$ T Cells.** In the absence of any intervention, at 21 days following the injection of tumor cells, multiple pulmonary gross and microscopic pulmonary metastases were evident (data not shown). Those animals which received adoptive

transfer of tumor-reactive TGF- β -insensitive CD8 $^+$ T cells had the least degree of tumor burden (Fig. 4A and B). There was no evidence of pulmonary metastasis in the group of mice which received adoptive transfer at 3 days following the injection of tumor cells. One of 10 animals in the 7-day group was found to have microscopic evidence of pulmonary metastasis at the time of sacrifice. Two of nine animals in the 21-day group must be sacrificed earlier due to poor health conditions and were found to have pulmonary metastasis of the tumor. Animals which received adoptive transfer of tumor-reactive control CD8 $^+$ T cells (GFP only) showed an intermediate degree of tumor burden; whereas those which received adoptive transfer of naive CD8 $^+$ T cells were ineffective in inhibiting tumor progression. Analysis of Kaplan-Meier survival curve showed highly significant differences among three treatment groups (Fig. 4C).

Histologic Findings. The most prominent histologic feature of the tumor tissue in this study is the evidence of infiltration of CD8 $^+$ T cells into the tumor tissue and the presence of apoptosis in tumor cells of animals which received adoptive transfer of tumor-reactive TGF- β -insensitive CD8 $^+$ T cells (the T β RIIDN group; Fig. 6A). Tumors in animals of the other two groups showed little or no CD8 $^+$ T cells and showed no evidence of apoptosis in tumor cells (Fig. 6A). CD8 $^+$ T cells, however, are present in the parenchymal tissue of the lung in animals of all groups (Fig. 6A). Although CD8 $^+$ T cells were not observed in tumors of animals in the GFP group, immune cell infiltration was apparent according to histologic observation (Fig. 5E), but such immune cell infiltration was more prominent in tumor of the T β RIIDN group (Fig. 5F). No immune cell infiltration was noted in tumors of animals which received adoptive transfer of naive CD8 $^+$ T cells (Fig. 5D).

Another feature is the lack of infiltration of immune cells in the air space of the lung in all animals, including those which received adoptive transfer of tumor-reactive TGF- β -insensitive CD8 $^+$ T cells (Fig. 5). This is in sharp contrast to our earlier studies, in which recipient animals which received TGF- β -insensitive bone marrow transplant and developed massive infiltration of immune cells in the air space of the lung (19, 20). In the present study, the air space was devoid of any immune cells, suggesting that autoimmune disease was not apparent in these animals.

Fate of Transferred CD8 $^+$ T Cells. In the present study, a total of 2×10^6 CD8 $^+$ T cells were transferred into each recipient animal. This number was derived by extrapolating the comparable number of T cells in adoptive therapy for cancer patients (1, 6). In a similar study with adoptive transfer of experimental melanoma model, 3×10^6 antigen-specific T cells were used (21). To determine the fate of these transferred cells, we measured the percentage of GFP-positive CD8 $^+$ T cells in the spleen of recipient animals. When CD8 $^+$ T cells were adoptively transferred to tumor-free hosts, there was a linear decay in GFP-positive CD8 $^+$ T cells reaching 0% by 30 days for tumor-reactive CD8 $^+$ T cells infected with the GFP control vector and 50 days for tumor-reactive CD8 $^+$ T cells infected with the T β RIIDN vector (Fig. 6B). However, when tumor-reactive TGF- β -insensitive CD8 $^+$ T cells were adoptively transferred to tumor-bearing hosts (the T β RIIDN group), the percentage of GFP-positive CD8 $^+$ T cells was maintained at 2% for at least 40 days; whereas in animals which received adoptive transfer of tumor-reactive control CD8 $^+$ T cells (the GFP group), GFP-positive CD8 $^+$ T cells showed a decay curve similar to that in tumor-free hosts with a slight delay (Fig. 6C).

Serum Levels of IFN- γ and IL-2. In animals which received adoptive transfer of naive CD8⁺ T cells, there was a baseline level of IL-2 and IFN- γ . In animals which received tumor-reactive control CD8⁺ T cells (the GFP group), there was a significant increase in both cytokines. A further increase in serum IL-2 (Fig. 6D) and IFN- γ (Fig. 6E) was observed when these cells were rendered insensitive to TGF- β (the T β RIIDN group), suggesting the presence of activated immune cells in these hosts.

Discussion

Immunotherapy using adoptive transfer of immune cells is a promising approach for treatment of cancer patients. However, currently available therapies have not achieved a significant number of complete responders. A successful adoptive therapy for cancer should be the development of robust effector cells with specific antitumor efficacy. At the same time, the treatment will overcome the tumor-derived immunosuppressive effect.

A significant part of tumor immunology has focused on the identification of tumor-specific antigens and the cytolytic T cells specific for these peptides (22). Adoptive T-cell therapy using antigen-specific CD8⁺ T cells for cancer treatment has been attempted with some degree of success (5, 23) and seems to be an advantage over the transfer of nonspecific T cells (21). Results of the present study have shown that adoptive transfer of tumor-reactive and TGF- β -insensitive CD8⁺ T cells were able to specifically target against autologous tumor cells and eradicate established pulmonary metastasis. The use of tumor-specific adoptive immunotherapy has reported before and has clearly shown its efficacy by other investigators (23, 24). The critical issue in immunotherapy thus far has been the tumor-derived immunosuppressive effect, which remains unresolved.

The mouse prostate cancer model, TRAMP-C2, represents an aggressive line of malignant cells, which secrete large amounts of TGF- β . The role of immunosuppressive effect of TGF- β in cancer progression has been well established (13, 25–27). In the present study, we have shown that, TRAMP-C2 tumors possess potent immunosuppressive power so that regular CD8⁺ T cells are unable to infiltrate into the tumor tissues. However, if these tumor-reactive CD8⁺ T cells are engineered and rendered insensitive to TGF- β , they are able to infiltrate into the tumor tissue and induce apoptosis in these established TRAMP-C2 tumors. To the best of our knowledge, studies to test this concept have not been attempted before. These results support the concept that TGF- β is an important target in cancer therapy.

Results of the present study show that in tumor-bearing hosts, the transferred CD8⁺ T cells persist, only if they are tumor reactive and TGF- β insensitive. Therefore, adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells will persist in tumor-bearing hosts and does not require the procedure of lymphodepletion. Interestingly, these cells decayed in tumor-free hosts. Accompanied with the persistence of these transferred CD8⁺ T cells were elevated circulating levels of IL-2 and IFN- γ , a critical requirement for antitumor activity in the host (24). Therefore,

with our current approach of adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells, exogenous treatment of IL-2 is not necessary for a successful antitumor activity. On the other hand, naive CD8⁺ T cells and tumor-reactive CD8⁺ T cells but sensitive to TGF- β did not persist in the host, suggesting that these cells failed to establish an engraftment regardless the status of the presence or absence of tumor cells in the host. These observations suggest that a single transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells is sufficient for tumor rejection.

Our results also indicate that CD8⁺ T cells contain high levels of TGF- β receptor types I and II and therefore, are highly sensitive to the inhibitory effects of TGF- β . The role of TGF- β in the immune system is best shown in TGF- β knockout animals. Mice lacking TGF- β , although they grew normally for the first 2 weeks, develop rapid wasting syndrome, and die by 3 to 4 weeks of age (28, 29). These studies showed a powerful immunoregulatory role of TGF- β because TGF- β ^{-/-} mice had excessive inflammatory responses with massive infiltration of lymphocytes and macrophages in multiple organs. These syndromes were characterized as autoimmunity (17, 29). Results of our past study have shown that mice receiving TGF- β -insensitive bone marrow transplants have met with the same fate by developing autoimmune syndrome, although these animals were able to eliminate challenged tumors (19, 20). In the present study, the use of the tumor-specific TGF- β -insensitive CD8⁺ T cells for the treatment of established cancer did not result in the development of massive infiltration of immune cells into the airspace of the lung of tumor-bearing host and, in tumor-free hosts, these CD8⁺ T cells failed to persist in the host. These preliminary observations seem to suggest an apparent absence of the development of autoimmune disease in these animals. Further studies are warranted to verify this impression.

In summary, the present results showed that adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells to tumor-bearing hosts was able to eradicate autologous tumors. These CD8⁺ T cells have the following characteristic properties. First, they are specifically reactive against tumor tissues. Second, they are insensitive to TGF- β . These two properties endowed these CD8⁺ T cells with the ability to infiltrate into tumor tissues and function as potent effectors against tumor cells. Finally, these cells are able to persist in tumor-bearing hosts but not in tumor-free hosts. These findings provide a proof of principle that an adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells may warrant consideration for the treatment of advanced cancers.

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Infiltration of Tumor-Reactive Transforming Growth Factor-Beta Insensitive CD8⁺ T Cells Into the Tumor Parenchyma is Associated With Apoptosis and Rejection of Tumor Cells

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BACKGROUND. TGF- β is a potent immunosuppressant. High levels of TGF- β produced by cancer cells have a negative inhibition effect on surrounding host immune cells and leads to evasion of the host immune surveillance and tumor progression. In the present study, we report a distinct ability of tumor reactive, TGF- β -insensitive CD8⁺ T cells to infiltrate into established tumors, secrete relevant cytokines, and induce apoptosis of tumor cells.

METHODS. CD8⁺ T cells were isolated from the spleens of C57BL/6 mice, which were primed with irradiated mouse prostate cancer cells, the TRAMP-C2 cells. After ex vivo expansion, these tumor reactive CD8⁺ cells were rendered TGF- β -insensitive by infection with a retroviral (MSCV)-mediated dominant negative TGF- β type II receptor (T β RIIDN). Control CD8⁺ cells consist of those transfected with the GFP-only empty vector and naïve CD8⁺ T cells. Recipient mice were challenged with a single injection of TRAMP-C2 cells 21 days before adoptive transfer of CD8⁺ T cells was performed. Forty days after the adoptive transfer, all animals were sacrificed. The presence of pulmonary metastases was evaluated pathologically. Serial slides of malignant tissues were used for immunofluorescent staining for different kinds of immune cell infiltration, cytokines, and apoptosis analysis.

Abbreviations: TGF- β , transforming growth factor beta; GFP, green fluorescent protein; T β RIIDN, dominant-negative type II TGF- β receptor; TRAMP, transgenic adenocarcinoma of the mouse prostate; IFN- γ , interferon-gamma; IL-2, interleukin-2; NO, nitric oxide; TNF- α , tumor necrosis factor-alpha; PCNA, proliferating cell nuclear antigen; NK, natural killer cells; TR, Texas red; FITC, fluorescein isothiocyanate.

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RESULTS. Pulmonary metastases were either eliminated or significantly reduced in the group receiving adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells (3 out of 12) when compared to GFP controls (9 out of 12), and naïve CD8⁺ T cells (12 out of 12). Results of immunofluorescent studies demonstrated that only tumor-reactive TGF- β -insensitive CD8⁺ T cells were able to infiltrate into the tumor and mediate apoptosis when compared to CD4⁺ T cells, NK cells, and B cells. A large amount of cytokines such as perforin, nitric oxide, IFN- γ , IL-2, TNF- α were secreted in tumor tissue treated with tumor-reactive TGF- β -insensitive CD8⁺ T cells. No immune cells infiltration and cytokine secretion were detected in tumor tissues treated with naïve T cells and GFP controls.

CONCLUSIONS. Our results demonstrate the mechanism of anti-tumor effect of tumor-reactive TGF- β -insensitive CD8⁺ T cells that adoptive transfer of these CD8⁺ T cells resulted in infiltration of these immune cells into the tumor parenchyma, secretion of relevant cytokines, and induction of apoptosis in tumor cells. These results support the concept that tumor-reactive TGF- β -insensitive CD8⁺ T cells may prove beneficial in the treatment of advanced cancer patients. *Prostate* 66: 235–247, 2006. © 2005 Wiley-Liss, Inc.

KEY WORDS: TGF- β ; adoptive transfer; gene therapy; CD8⁺ T cell; immunosurveillance; tumor rejection

INTRODUCTION

Immunotherapy using adoptive transfer of immune cells is a promising approach for treating cancer patients. The presence of tumor infiltrating lymphocytes (TIL) in the tumor parenchyma has been recognized for three decades [1]. TIL were isolated from surgical specimens, clonally expanded ex vivo, and adoptively transferred to cancer patients with variable results [2,3]. Recently, Yee and coworkers selected antigen-specific CD8⁺ T cells for ex vivo expansion and transferred these cells into patients. However, these CD8⁺ T cells did not persist, requiring repeated transfers of CD8⁺ T cells in order to elicit clinical responses [4]. Rosenberg and colleagues treated autologous TIL cells with IL-2 for ex vivo expansion and then transferred them to patients. Again, in order for these cells to “engraft,” lymphodepletion was necessary [5]. These results, although impressive, seem to suggest a missing element in adoptive transfer of CD8⁺ T cells for the treatment of cancer.

The functional role of TIL in cancer therapy has been a subject of controversy [6]. Initially, it appeared that the presence of TIL in tumors could correlate to prognosis [7]. Subsequent studies showed that TIL were functionally impaired [8–10] from effects exerted by the tumor microenvironment [11]. The development of an immune-based strategy for cancer therapy must take into account not only immune stimulation, but also the issue of overcoming tumor-derived immune suppression [12]. Among many immunosuppressants, TGF- β is a potent and important player [13–18].

High levels of TGF- β produced by cancer cells have a negative effect on surrounding cells such as the host immune cells and have been implicated to play a role in tumor escape from immune surveillance [19,20]. Besides the tumor, the immune system, in response to

the presence of tumor, is also able to produce a significant amount of TGF- β to down-regulate immune surveillance [21].

TGF- β , therefore, appears to be an attractive target for anti-cancer therapy. Investigators have attempted to utilize the properties of TGF- β advantageously for the treatment of cancer. Gorelik and Flavell [17] first described the immune-mediated eradication of tumors through the blockade of TGF- β signaling in T cells. These investigators used transgenic mice with TGF- β null expression targeted specifically to T cells. Subsequently, our study using transplant of TGF- β -insensitive bone marrow cells also demonstrated a total rejection of metastatic tumor cells [22,23]. However, autoimmune disease eventually developed in the hosts in both studies due to the non-specific nature of the immune cells. More over recently, we have shown that adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells, which have high specific tumor killing ability and were able to eradicate established lung metastases of mouse prostate cancer cells, TRAMP-C2, which secreted large amounts of TGF-beta [24]. In the present study, we conducted additional experiments to delineate the mechanism of the tumoricidal ability of tumor-reactive TGF- β -insensitive CD8⁺ T cells. We report a distinct ability of these CD8⁺ T cells to infiltrate into established tumors, secrete relevant cytokines, and induce apoptosis of tumor cells.

MATERIALS AND METHODS

Mice and Cells

Male C57BL/6 mice of 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University

Feinberg School of Medicine in accordance with the established guidelines of the Animal Care and Use Committee of Northwestern University. TRAMP-C2 is an early-passage murine prostate cancer cell line derived from TRAMP mice that spontaneously develops prostate cancer due to prostate-specific simian virus 40 (SV 40) large T tumor antigen (Tag) expression. Cells were cultured in RPMI-1640 medium (GIBCO, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (GIBCO).

Generation of Tumor-Reactive TGF- β -Insensitive CD8⁺ T Cells

Ex vivo expansion of tumor-reactive CD8⁺ T cells from the splenocytes: Male C57BL/6 mice were primed with irradiated TRAMP-C2 cells (5×10^6 /mice at 20,000 ci) by subcutaneous injection every 14 days for a total of five inoculations. Two weeks following the last vaccination, CD8⁺ T cells from the spleen were isolated by using murine T cell CD8 subset column kit (R&D Systems, Minneapolis, MN). CD8⁺ T cells (10^5 /ml) were cultured in the presence of TRAMP-C2 lysates (1×10^6 /ml) and irradiated mouse splenocytes (1×10^6 /ml at 3,000 ci) in medium containing RPMI-1640 with 10% FBS, IL-2 (50 U/ml, R&D), CD3⁺ monoclonal antibody (30 ng/ml, R&D), HEPE (25 mM), L-glutamine (4 mM), and 2-ME (25 mM) (Sigma, St. Louis, MO) at 37°C and under 5% CO₂. Culture media were changed every 3 days. CD8⁺ T cells were cultured for around 10 weeks before they were infected with a retrovirus containing dominant negative TGF- β type II receptor (T β RIIDN-GFP) vector or the control GFP only vector. Rendering insensitivity to TGF- β by infection with T β RIIDN-GFP-containing retrovirus: Construction of the mouse stem cell retroviral vector (MSCV) containing the dominant negative TGF- β type II receptor (T β RIIDN) and green fluorescent protein (GFP) was performed as previously described [22,23]. Tumor-reactive CD8⁺ T cells above were infected with retroviral particles containing T β RIIDN-GFP or GFP only via spin infection as described earlier [22,23]. Infection efficiency was assessed for GFP expression by flow cytometry, only the infection efficiency was up to 90%, the cells could be used for adoptive transfer that was performed 72 hr after infection.

Tumor Bearing Animal Survival Analysis

Male C57BL/6 mice were challenged i.v. with 5×10^5 TRAMP-C2 cells. Twenty-one days later, they received adoptive transfer of one of the three groups of CD8⁺ T cells (2×10^6). CD8⁺ T cells in Group 1 were tumor-reactive TGF- β -insensitive CD8⁺ T cells infected with the T β RIIDN-GFP viral particles. Those in Group 2

were tumor reactive CD8⁺ T cells infected with the GFP-only control vectors. Cells in Group 3 were naive CD8⁺ T cells. Forty days after CD8⁺ T cells transfer, mice were sacrificed by cervical dislocation. Some animals were sacrificed earlier than 40 days due to poor health conditions. The lung from each animal was isolated for gross and histological examination. At 40 days, all animals were inspected for the presence of pulmonary metastases. The time of sacrifice for mice in each group was compared by the Kaplan–Meier method. The pulmonary specimens were prepared as serial sections with 4 μ m for each through the whole lungs.

Immunohistochemical Staining

H&E staining: Upon euthanasia, the lung from each animal was excised, fixed in formalin, embedded in paraffin, and serially sectioned at 4 μ m thick until the embedded tissue was exhausted. Routine Hematoxylin & Eosin (H&E) staining was performed at an interval of every five serial sections. All H&E sections were evaluated for the presence of tumor tissues by at least three independent investigators. PCNA immunological staining: VECTASTAIN ABC kit (Vector Labs, Burlingame, CA) was used according to the procedure from the manufacturer with the following adjustment: after deparaffinization, quenching of endogenous peroxidase activity and normal serum pre-blocking, the sections were incubated in diluted mouse monoclonal PCNA antibody (1:100, Upstate, Lake Placid, NY) for 2 hr in room temperature, followed by incubation with biotinylated goat horse anti-mouse secondary antibody (1:200, Vector Labs) for 2 hr. Then peroxidase substrate solution DAB (DAKO Corporation, Carpinteria, CA) was used for desired staining and Harris Hematoxylin Solution for counterstaining.

Immunofluorescent co-Staining for Infiltration of Immune Cells and Secretion of Cytokines

Unstained paraffin-embedded serial sections of metastatic cancer to the lung were used for immunofluorescent staining to detect infiltration of immune cells (CD8⁺ T, CD4⁺ T, B cells, and NK cells) and secretion of cytokines (perforin, Nitric Oxide, IFN- γ , TNF- α , IL-2). The methods of immunofluorescent co-staining were performed by using the assay as previously described [24]. (A): Expression of TGF- β in tumor tissue was analyzed by using Nuclear-TGF- β 1 double staining; (B): Infiltration of immune cells in tumor tissue was analyzed by Nuclear-immune cells double staining. Mouse CD8⁺ T cells, CD4⁺, NK(NCAM) cells, B (BLCAM) cells were evaluated, respectively; (C): Identification of the source of CD8⁺ T cells in tumor tissue: Nuclear-CD8⁺-GFP protein triple staining; (D): Secretion of cytokines were analyzed by:

Nuclear-CD8⁺-cytokines triple staining. Expression of perforin, Nitric Oxide, IFN- γ , TNF- α , IL-2 were analyzed. The parameters of the antibodies (Santa Cruz, Santa Cruz, CA) are listed in Table I. All the slides were deparaffinized and blocked by normal serum. The sections were then incubated with the fluorescent staining as described before [23]. All the slides were stained with VECTASHIELD mounting media (blue) (Vector lab) for nuclear counterstaining. Staining was viewed with Nikon TE2000-U fluorescent microscopy (Nikon Corporation, Tokyo, Japan). Images were digitized by Photoshop 7.0 with a PC computer. The intensity of the fluorescent signal was standardized by the standard fluorescent index (positive lymphocytes or signal/100 tumor cells/1,000 μm^2): -: <5; \pm : 6–10; +: 11–30; ++: 31–50; +++: 51–70; ++++: >70.

Immune-Mediated Tumor Apoptosis Assay

Following deparaffinizing, tissue sections were subjected to apoptosis assay by using the TUNEL apoptosis kit (Upstate, Lake Placid, NY) according to the recommendations of the manufacturer. Briefly, the slides were treated with Proteinase K for 30 min at 37°C, incubated with TdT end-labeling cocktail (TdT Buffer, Biotin-dUTP, and TdT, at a ratio of 90:5:5) for 120 min at 37°C followed by Avidin-FITC (green) solution (50 μl), incubated in the dark for 60 min at 37°C. Slides were then incubated with 50 μl of blocking buffer at room temperature for 20 min followed by rat monoclonal antibody for CD8⁺ labeled with TR (red) (2 $\mu\text{g}/\text{ml}$, Santa Cruz) in the dark for 30 min at room temperature. Finally, slides were washed with PBS and stained with VECTASHIELD mounting media (blue) (Vector lab) for nuclear staining. They were viewed with Nikon TE2000-U fluorescent microscopy (Nikon Corporation, Tokyo, Japan). Images were digitized by Photoshop 7.0 with a PC computer.

Statistical Analysis

Analysis of variance and multiple range tests were performed to determine differences of means among different treatment groups. A *P*-value of less than 0.05 was considered statistically significant. SPSS 10.0.7 software package (SPSS Inc.) was used for analysis. Kaplan–Meier survival curve was analyzed by the log-rank test using the Graphpad Prism 4.02 software (Graphpad Software Inc., San Diego, CA).

RESULTS

In Vivo Anti-Tumor Activity of Tumor Reactive, TGF- β -Insensitive CD8⁺ T Cells

To demonstrate the in vivo anti-tumor activity, male C57BL/6 mice of 6–8 weeks old were challenged with

5×10^5 TRAMP-C2 cells by intravenous injections for 21 days. In the absence of any intervention, at 21 days following tumor cell challenge, multiple gross and microscopic pulmonary metastases were evident. All animals were sacrificed 40 days following CD8⁺ T cells transfer, or sooner due to poor health conditions. Mice who received tumor-reactive, TGF- β -insensitive CD8⁺ T cells had the least degree of tumor burden. Nine of 12 animals were free of pulmonary metastasis (Fig. 1A) and 3 mice with metastasis had an average number of metastasis per animal (number \pm SD) of 1.67 ± 0.5 . Animals who received tumor reactive control CD8⁺ T (GFP only) cells showed partial anti-tumor activity with 3 out of 12 animals showing tumor-free and those with metastasis had an average number of metastasis per animal (number \pm SD) of 3.7 ± 1.1 . Adoptive transfer of naïve CD8⁺ T cells was ineffective in inhibiting tumor progression. All animals in the group had pulmonary metastasis with the average number of metastasis per animal (number \pm SD) being 4.8 ± 1.5 . Results of Kaplan–Meier analysis showed significant survival differences among the three treatment groups (Fig. 1B).

Histological Findings

The H&E stained sections of tumors that metastasized to the lung were evaluated (Fig. 2A,C,E with magnification of 100 \times ; 2B, 2D, and 2F with a magnification of 400 \times). In tumors from animals who received naïve CD8⁺ T cells, a portion of a large tumor (Fig. 2A and 3 mm in diameter) with marked cytological polymorphism (Fig. 2B) is illustrated. In the mouse who received adoptive transfer of tumor-reactive control CD8⁺ T cells (GFP only), there is one smaller tumor nodule (Fig. 2C, 1.5 mm in diameter), which demonstrates some infiltration and degenerative changes of tumor cells (Fig. 2D). In the tumor from mice who received adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells (T β RIIDN), there is a smaller tumor nodule (Fig. 2E, 0.5 mm in diameter) with heavy lymphocytic infiltrates and marked degenerative changes of tumor cells (Fig. 2F). Another feature is the lack of infiltration of immune cells in the air spaces of the lung in all animals, including those who received adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells, suggesting that autoimmune disease was not apparent in these animals. This contrasts to our earlier study in which recipient animals who received TGF- β insensitive bone marrow transplant developed massive infiltration of immune cells in the air spaces of the lung [22,23].

A tumor nodule in a mouse who received naïve CD8⁺ T cells, composed of tumor cells with high proliferative activity (>80% cells positive for PCNA) is shown in Figure 2G,H. In animals who received

TABLE I. Antibodies for Immunofluorescent Staining

A. Nuclear-TGF-β1 double staining				
TGF-β1 staining			Nuclear staining	
TGF-β1	Primary antibody Polyclonal-rabbit-mouse, 1: 100, 2 hr	Secondary antibody Goat-rabbit-FITC, 1:300, 2 hr	VECTASHIELD mounting medium with DAPI	
B. Nuclear- immune cells double staining				
CD8+ T cells (CD8-2.43)	Immune cells staining Monoclonal-rabbit-mouse-FITC, 1:300, 2 hr	Nuclear staining VECTASHIELD mounting medium with DAPI		
CD4+ T cells (CD4-RM4 5)	Monoclonal-rabbit-mouse-FITC, 1:300, 2 hr			
B cells (BLCAM-H221)	Monoclonal-rabbit-mouse-FITC, 1:300, 2 hr			
NK cells (NCAM-H300)	Monoclonal-rabbit-mouse-FITC, 1:300, 2 hr			
C. Nuclear-CD8 ⁺ -GFP protein triple staining				
Monoclonal mouse Ig G	GFP rhodamine 1:100, 2 hr/RT	CD8 ⁺ staining Monoclonal-rat-mouse-FITC 1:100, 2 hr/RT	Nuclear staining VECTASHIELD mounting medium with DAPI	
D. Nuclear-CD8 ⁺ - cytokines triple staining				
	Cytokines staining		CD8 ⁺ staining	Nuclear staining
Perforin	Primary antibody Polyclonal-rabbit-mouse, 1:100, 2 hr	Secondary antibody Goat-rabbit-FITC, 1:500, 2 hr	Monoclonal-rat-mouse-TR, 1:100, 2 hr	VECTASHIELD mounting medium with DAPI
IFN-γ	Polyclonal-goat-mouse, 1:150, 2 hr	Donkey-goat-FITC, 1:250, 1.5 hr	Monoclonal-rat-mouse-TR, 1:100, 2 hr	
Nitric oxide	Monoclonal-mouse-mouse, 1:100, 2 hr	Goat-mouse- TR, 1:500, 2 hr	Monoclonal-mouse-mouse-FITC, 1:100, 2 hr	
IL-2	Polyclonal-rabbit-mouse, 1:100, 2 hr	Goat-rabbit-FITC, 1:300, 2 hr	Monoclonal-rat-mouse-TR, 1:100, 2 hr	
TNF-α	Polyclonal-rabbit-mouse, 1:100, 1 hr	Goat-rabbit-FITC, 1:300, 2 hr	Monoclonal-rat-mouse-TR, 1:100, 2 hr	

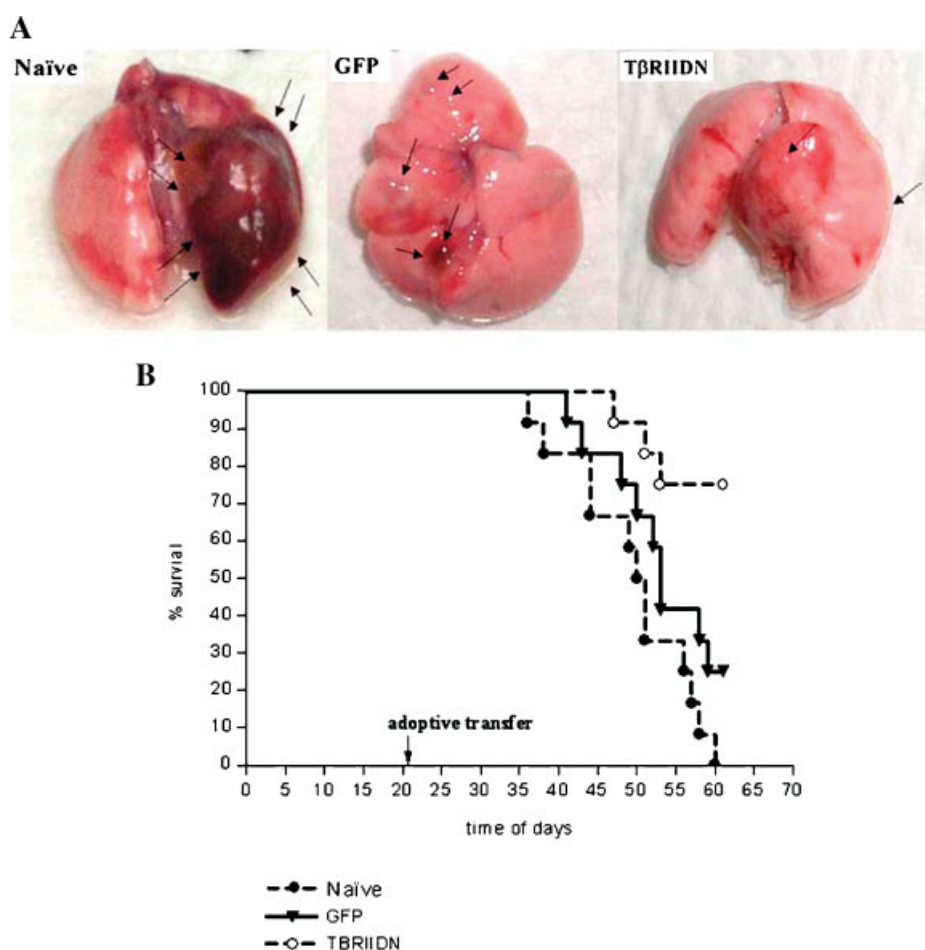


Fig. 1. In vivo anti-tumor activity of tumor reactive TGF- β -insensitive CD8⁺ T cells. **A:** Status of pulmonary metastasis of TRAMP-C2 tumors in mice received adoptive transfer of CD8⁺ T cells. There are three types of CD8⁺ T cells: Naïve CD8⁺ T cells were CD8⁺ T cells isolated from spleens of untreated C57BL/6 mice, tumor-reactive control CD8⁺ T cells (GFP), and tumor-reactive TGF- β -insensitive CD8⁺ T cells (T β RIIDN). TRAMP-C2 cells (5×10^5) were challenged to recipient mice. At 21 days following tumor challenge, animals were sacrificed at 40 days following the adoptive transfer or sooner due to poor health conditions. Representative gross feature of lung tissues from tumor-bearing mice at 40 days following tumor challenge. Arrows indicate metastatic sites. **B:** Kaplan-Meier survival curve of tumor-bearing mice received adoptive transfer of naïve CD8⁺ T cells, GFP-infected CD8⁺ T cells, and T β RIIDN-infected CD8⁺ T cells. $P < 0.05$ by the log-rank test for the T β RIIDN group versus the naïve group or GFP group.

tumor-reactive control CD8⁺ T cells (the GFP group), the majority of tumor cells show high proliferative activity (>80% cells positive for PCNA) (Fig. 2I,J). On the contrary, the degenerative tumor cells in mice receiving tumor-reactive TGF- β -insensitive CD8⁺ T cells show low proliferative activity (<10% cells positive for PCNA) (Fig. 2K,L). There is no statistic difference between naïve group and GFP group, but there is a significant difference between T β RIIDN groups and the above two groups ($P < 0.05$).

Infiltration of Tumor-Reactive TGF- β -Insensitive CD8⁺ T Cells in Tumor Tissue

The most prominent histological feature of the tumor tissue in this study is evidence of infiltration of

large amounts of tumor-reactive TGF- β -insensitive (GFP staining positive) CD8⁺ T cells and the presence of apoptosis in tumor tissues (Fig. 3A). Almost all of these infiltrated CD8⁺ T cells are GFP positive, suggesting that all these CD8⁺ T cells were adoptively transferred (Fig. 3E). Also, CD4⁺, B cells, and NK cells, thought not abundant, were found in the tumor tissue (Fig. 3B–D). Tumors in animals who received adoptive transfer of naïve CD8⁺ T cells and tumor-reactive control CD8⁺ T cells (Fig. 3A) showed little or no CD8⁺ T cells. In the latter two groups, CD8⁺ T cells are present only in the stromal tissues of the lung. Although CD8⁺ T cells were not observed in tumors of animals who received tumor-reactive control CD8⁺ T cells, some lymphocytic infiltration was evident histologically. However, such lymphocytic infiltration was more

prominent in tumors of the group who received tumor-reactive TGF- β -insensitive CD8⁺ T cells. Very little CD4⁺ T cells, B cells, and NK cells were observed in the tumor tissue of the tumor-reactive control CD8⁺ T cells treatment group. No lymphocytic infiltration was

noted in tumors of animals who received naïve CD8⁺ T cells (Fig. 3A,B,D), except some B cells (Fig. 3C). The degree of infiltration of different kinds of lymphocytes were evaluated by standard fluorescent index, which corresponds to the fluorescent intensity criterion (positive lymphocytes/100 tumor cells/1,000 μm^2 : - : <5; \pm : 6–10; +: 11–30; ++: 31–50; +++: 51–70; ++++: >70) (Fig. 3G). In general, much higher TGF- β expression was detected in the tumor parenchyma than peripheral non-tumor tissue in all three groups (Fig. 3F).

Secretion of Cytokines in Tumor Tissue

A very high level of perforin (Fig. 4A), along with an abundance of infiltrated tumor-reactive TGF- β -insensitive (GFP staining positive) CD8⁺ T cells was expressed in tumor tissue. The superimposed image suggests that the overwhelming majority of perforin originated from tumor-reactive TGF- β -insensitive CD8⁺ T cells. A marginally high level of IFN- γ (Fig. 4B) and IL-2 (Fig. 4D) and a moderately high level of nitric oxide (Fig. 4C) were expressed in tumor tissue. IFN- γ , IL-2, and nitric oxide appeared to have originated primarily from tumor-reactive TGF- β -insensitive CD8⁺ T cells. Low levels of TNF- α expression were demonstrated in tumor tissue. The majority of TNF- α expression appeared to have originated from tumor-reactive TGF- β -insensitive CD8⁺ T cells (Fig. 4E). Increased levels of IFN- γ and IL-2 in tissue corresponded to serum levels (data now shown). Perforin was not expressed in naïve CD8⁺ T cells and was expressed very minimally in tumor-reactive control CD8⁺ T cells (Fig. 4A). The contribution of IFN- γ , IL-2, nitric oxide, and TNF- α expression from naïve CD8⁺ T cells and tumor-reactive control CD8⁺ T cells appears to be negligible (Fig. 4B). A summary of cytokine expression was described as

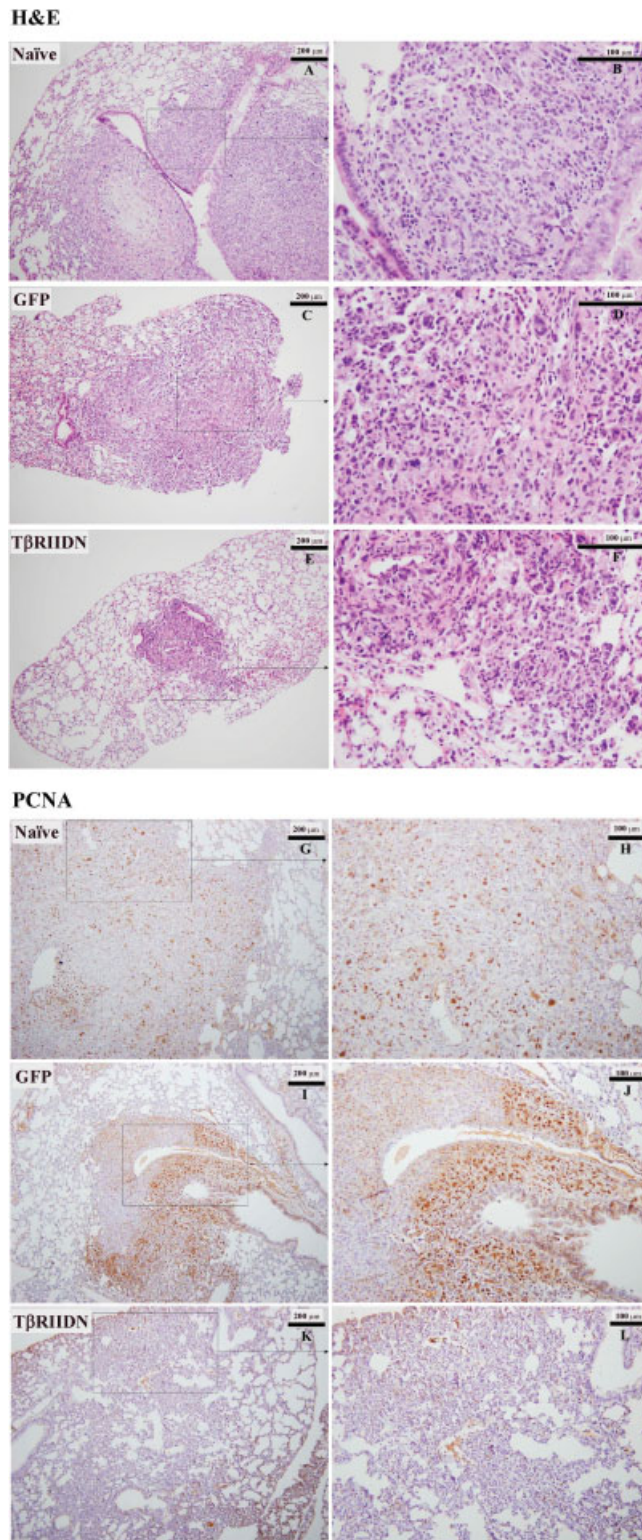


Fig. 2. Histological finding of the tumor. Representative histology (H&E staining) of metastatic tumor nodules in the lungs from animals received adoptive transfer of naïve CD8⁺ T cells (**A** and **B**), GFP-infected CD8⁺ T cells (**C** and **D**), and T β RIIDN-infected CD8⁺ T cells (**E** and **F**), in mice 40 days following adoptive transfer of CD8⁺ T cells into recipients at 21 days following injection of tumor cells. In the lung of a mouse that received transfer of naïve CD8⁺ T cells, a portion of a large tumor (**A**, 3 mm in diameter) showing marked cytological polymorphism is illustrated. In the lung of a mouse transferred with GFP-infected CD8⁺ T cells, there is one smaller tumor nodule (**C**, 1.5 mm in diameter), which demonstrates some degree of lymphocytic infiltration and degenerative changes of tumor cells. In the lung of a mouse that received T β RIIDN-infected CD8⁺ T cells, there is a smaller tumor nodule (**E**, 0.5 mm in diameter). Within this tumor, heavy lymphocytic infiltrates and marked degenerative changes of tumor cells can be seen (**F**). Immunohistological staining for the expression of PCNA in tumor cells: naïve (**G**, **H**), GFP (**I**, **J**), T β RIIDN (**K**, **L**). The brown cells are PCNA-positive cells. All specimens were observed at 100-fold, 200-fold, or 400-fold magnification.

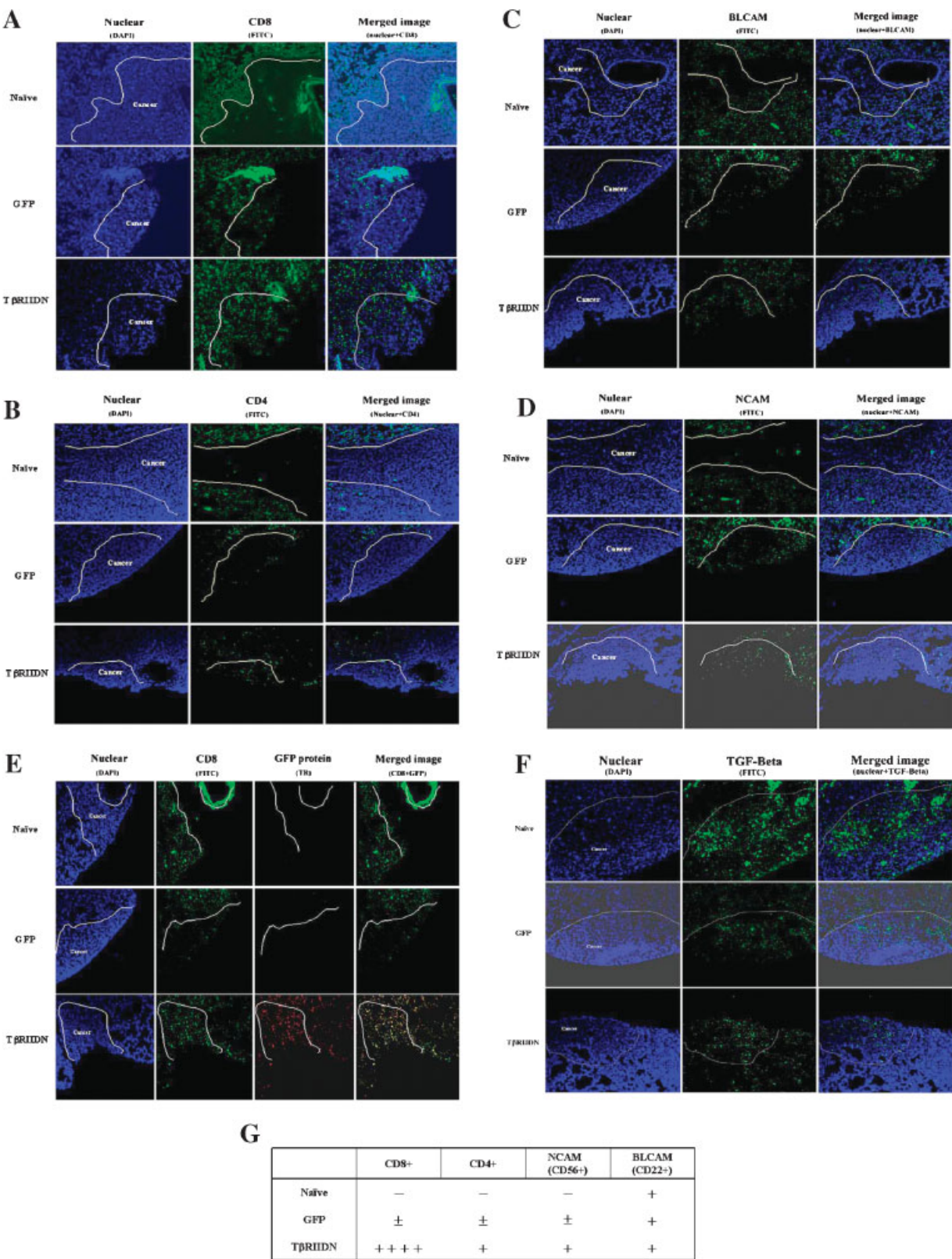


Fig. 3.

standard fluorescent index (positive signal/100 tumor cells/1,000 μm^2 : -: <5; \pm : 6–10; +: 11–30; ++: 31–50; +++: 51–70; ++++: >70) (Fig. 4F).

Apoptosis of Tumor Cells by the Treatment of Tumor-Reactive TGF- β -Insensitive CD8⁺ T Cells

A prominent feature of TUNEL assay in the tumor tissue in this study is the infiltration of CD8⁺ T cells into the tumor parenchyma and the presence of apoptosis in tumor cells of animals who received adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells (the T β RIIDN group) (Fig. 5). Tumors in the animals of the other two groups showed little or no infiltration of CD8⁺ T cells and demonstrated no evidence of apoptosis. However, CD8⁺ T cells are present in the stromal tissue of the lung in animals of all groups. Meanwhile, there are large amounts of CD8⁺ T cells that were induced to undergo apoptosis outside the margin of the tumor sites in animals treated with adoptive transfer of naïve CD8⁺ T cells and tumor-reactive control CD8⁺ T cells.

DISCUSSION

A significant part of modern tumor immunology has focused on the identification of tumor-specific antigens and the cytolytic T-cells specific for these peptides [25]. When such antigens are defined, therapeutic approaches use the antigen as the target [26]. However, any immunotherapeutic approach for cancer necessitates cytotoxic T lymphocytes to enter the tumor parenchyma. In the past, despite the ability to generate immune cells that are reactive against tumor antigens, evasion of the host immune surveillance by tumor cells persisted leading to eventual tumor progression [14,27,28]. Many possible mechanisms of a tumor's ability to evade host immune surveillance have been elucidated. These include the down-regulation of tumor antigen processing, the inhibitory role of CD4⁺CD25⁺ T regulatory cells, and the role of tumor-derived immunosuppressive cytokines, which include VEGF, IL-10, and TGF- β [28–32]. High levels of TGF- β produced by tumor cells are able to deter immune cells from entering the tumor parenchyma [33–38]. Results of the present study seem to indicate that TGF- β was a

very important immunosuppressant, as TGF- β insensitive CD8⁺ T cells were able to infiltrate into the tumor parenchyma and to induce apoptosis in tumor cells, and its immunosuppressive role in cancer progression has been well established.

The mouse prostate cancer, TRAMP-C2, represents an aggressive line of malignant cells, which secrete large amounts of TGF- β . Presently, we have demonstrated that TRAMP-C2 tumors, due to their secretion of large amounts of TGF- β [24], possess potent immunosuppressive power resulting in the inability of conventional CD8⁺ T cells to effectively infiltrate into the tumor tissue, resulting in a failure of these recipient animals to reject tumors. In this study, it is apparent that TGF- β -mediated evasion of the host immune surveillance system plays a critical role. Once these tumor-reactive CD8⁺ T cells were rendered insensitive to TGF- β , they possessed the ability to eradicate established pulmonary metastases in a mouse prostate cancer model and to prolong survival in tumor-bearing mice. We observed that adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells can overcome the tumor-derived immune suppressive mechanisms. The most prominent characteristic of these tumor-reactive TGF- β -insensitive CD8⁺ T cells is their ability to escape the immunosuppressive effect of tumor-derived TGF- β to specifically infiltrate into the tumor parenchyma, and to function as potent effectors against tumor cells that induce apoptosis in these established TRAMP-C2 tumors. Our experience has indicated that a mere acquisition of reactivity against tumor cells in CD8⁺ T cells was insufficient to achieve tumor infiltration by these cells [39,40]. Therefore, in order for tumor infiltration to occur, these CD8⁺ T cells must be rendered tumor-reactive as well as TGF- β -insensitive. Clinically, the large number of circulating tumor antigen-specific CD8⁺ cytotoxic T lymphocytes in individuals with cancer does not correlate with T-cell infiltration into cancer tissues or tumor regression [41,42]. The inability of immune cells, especially cytotoxic CD8⁺ T cell, to infiltrate into the tumor parenchyma is perhaps, the most important event in determining evasion from host immune surveillance by tumor cells. This is because all tumor cells have acquired the ability to inhibit the host immune system.

Fig. 3. Infiltration of lymphocytes into the tumor parenchyma. **A**: In contrast to GFP-infected and naïve CD8⁺ T cells, only T β RIIDN-infected CD8⁺ T cells can effectively infiltrate into the tumor parenchyma. Furthermore, unlike animals who were treated with T β RIIDN-infected CD8⁺ T cells, CD4⁺ T cells (**B**), and NCAM cells (**D**) except BLCAM cells (**C**) in animals treated with GFP-infected and naïve CD8⁺ T cells lacked the ability to effectively infiltrate into the tumor parenchyma. The source of CD8⁺ T cells within the tumor was confirmed to originate from T β RIIDN-infected CD8⁺ T cells that were adoptively transferred, as evidenced by nuclear-CD8⁺-GFP protein triple staining (**E**). When quantifying the relative contributions of the various immune cells and their ability to infiltrate into the tumor parenchyma, tumor-reactive TGF- β -insensitive CD8⁺ T cells contributed significantly, when compared to CD4⁺ T cells, BLCAM cells, and NCAM cells (**G**). In general, much higher TGF- β expression was detected in the tumor parenchyma than peripheral non-tumor tissue in all the three groups (**F**).

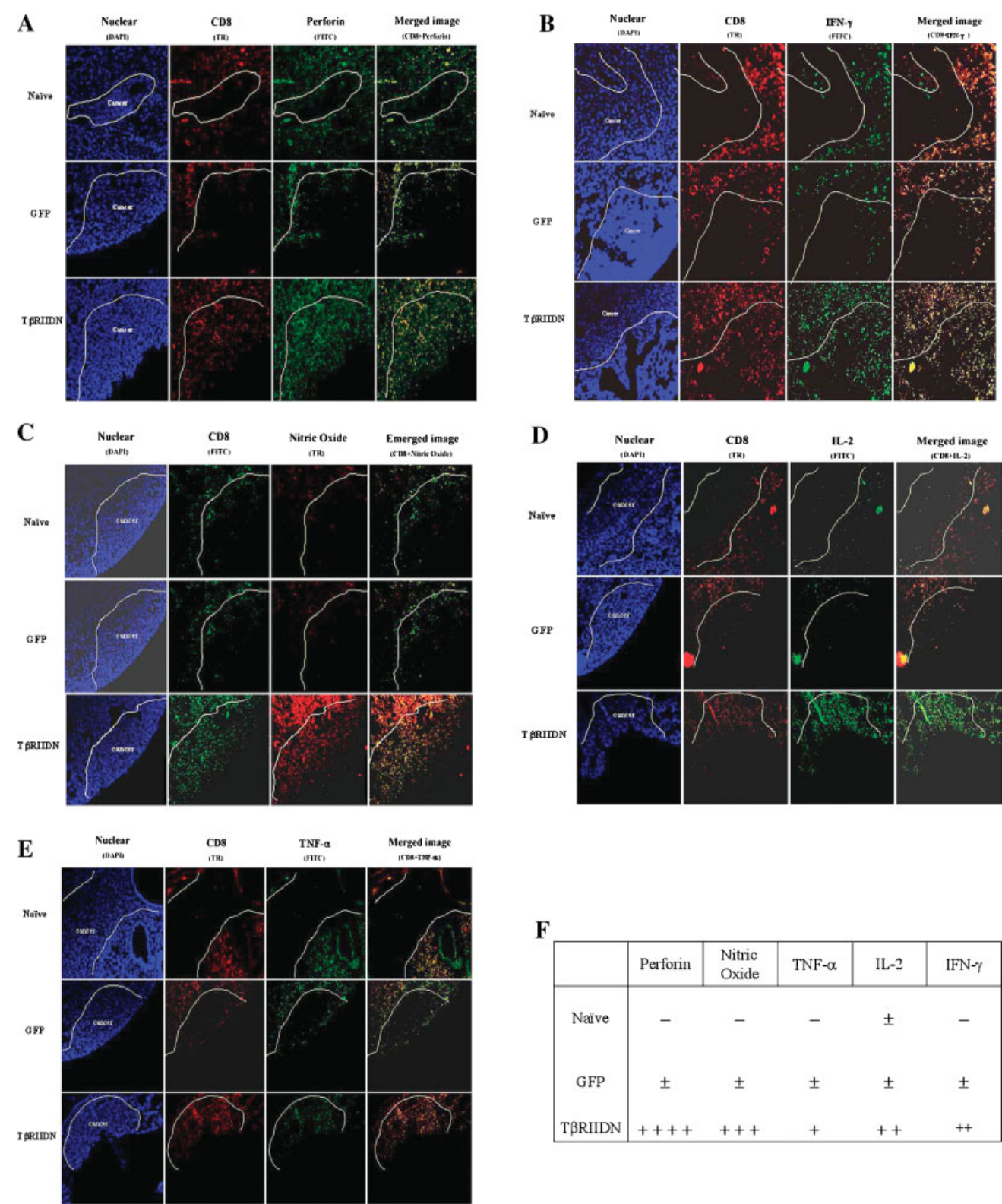


Fig. 4.

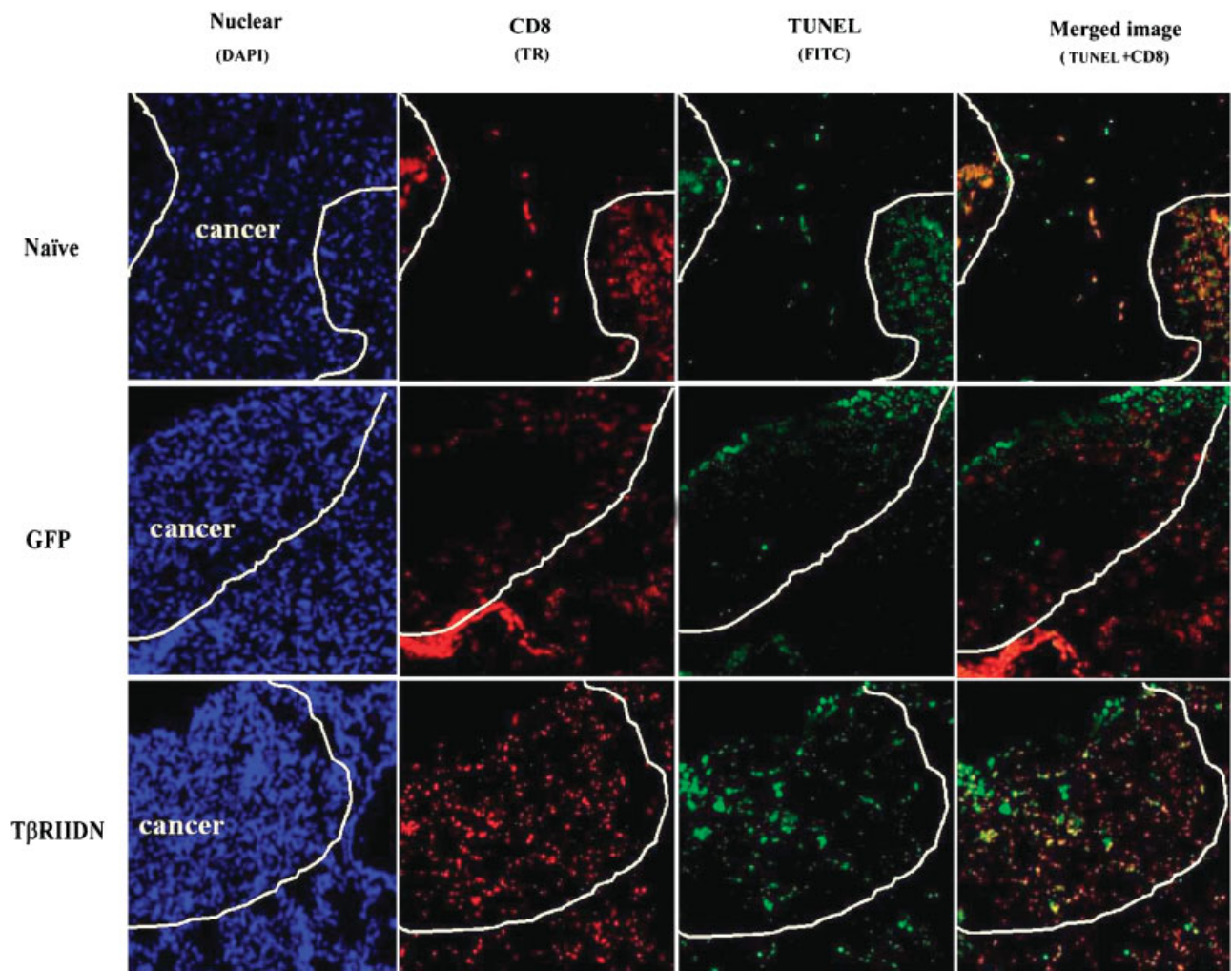


Fig. 5. Apoptosis of tumor cells. Immunofluorescent staining for nuclei, CD8⁺ T cells, and apoptosis. Representative tissue sections showing pulmonary metastasis were simultaneously stained for cell nucleus (blue), CD8⁺ T cells (red), and apoptosis (green). The tumor site was identified by the nuclear staining (blue). CD8⁺ T cells were identified mainly in the lung tissues not in the tumor with the exception of the TβRIIDN group, in which CD8⁺ T cells (red) were also found within the tumor parenchyma. Frequent tumor apoptotic sites (green) were only found in the TβRIIDN group. Although few CD8⁺ T cells were found undergoing apoptosis (yellow), the majority of the apoptotic cells were derived from the tumor cells (green). (magnification: $\times 400$) Apoptotic activity was evident within the tumor parenchyma of the TβRIIDN group. In contrast, tumors in animals who received treatments with GFP infected and naïve CD8⁺ T cells did not exhibit tumor cells apoptosis within the tumor parenchyma. Large amounts of CD8⁺ T cells exhibited apoptotic activity along the periphery of the tumor parenchyma.

Immunologists have studied properties of tumor cells and have recognized many factors that tumor cells have employed to inhibit the host immune system. These factors include soluble MHC class I chain-related (MIC)

molecules [43–45], cytotoxic T lymphocyte antigen-4 (CTLA-4) [46], interleukin-13, CD4⁺CD25⁺ regulatory T cells, and CD4⁺ natural killer T cells [47]. TGF- β has been recognized as one such tumor-derived immune

Fig. 4. Secretion of cytokines in tumors of different groups. **A:** A very high level of perforin is expressed in tumor tissue and corresponds to tumor-reactive TGF- β -insensitive CD8⁺ T cells that have effectively infiltrated into the tumor parenchyma. The merged image suggests that the overwhelming majority of perforin originated from tumor-reactive TGF- β -insensitive CD8⁺ T cells. Perforin was not expressed in naïve CD8⁺ T cells and expressed very minimally in GFP-infected CD8⁺ T cells. A marginally high level of IFN- γ (**B**), IL-2 (**D**), and a moderately high level of nitric oxide (**C**) corresponding to tumor-reactive TGF- β -insensitive CD8⁺ T cells is expressed in the tumor tissue. IFN- γ , IL-2, and nitric oxide appear to have originated primarily from tumor-reactive TGF- β -insensitive CD8⁺ T cells. The contribution of naïve and GFP-infected cells to IFN- γ , IL-2, and nitric oxide expression appears to be negligible. **E:** Low levels of TNF- α expression corresponding to tumor-reactive TGF- β -insensitive CD8⁺ T cells are demonstrated in the tumor tissue. The majority of TNF- α expression appears to have originated from tumor-reactive TGF- β -insensitive CD8⁺ T cells. The contribution of naïve and GFP-infected CD8⁺ T cells to TNF- α expression appears to be negligible. **F:** The relative contributions of the various cytokines were stratified according to different cell types.

suppressor [17]. Results of the present study suggest that TGF- β is a very important tumor-derived immune suppressor. In addition to their ability to infiltrate into the tumor parenchyma, tumor-reactive TGF- β -insensitive CD8⁺ T cells are readily activated [48], conferring them robust anti-tumor effector function, as indicated by the production of relevant cytokines and widespread apoptosis in tumor cells.

Once the TGF- β -insensitive cytotoxic T lymphocytes are allowed to infiltrate into the tumor parenchyma, these effector cells are able to launch powerful anti-tumor activities. Results of the present study indicated that large amounts of cytokines, including perforin, nitric oxide, IFN- γ , IL-2, TNF- α , were detected in tumor tissue. The infiltrated tumor-reactive TGF- β -insensitive CD8⁺ T cells produce these relevant cytokines, which mediate the tumor-killing activities. Lysis of tumor cells can be mediated by perforin, IFN- γ , IL-2, and nitric oxide [49]. The increased levels of IL-2 will also prolong persistence of transferred CD8⁺ T cells [4]. Therefore, tumors challenged with tumor reactive TGF- β -insensitive CD8⁺ T cells will be induced to undergo apoptosis. In contrast, tumors in those animals who received adoptive transfer of naïve CD8⁺ T cells or tumor-reactive control CD8⁺ T cells, survived as these CD8⁺ T cells were unable to infiltrate into the tumor parenchyma and were not allowed to interact with tumor cells. Without this "shield effect" of TGF- β , we observed a large amount of CD8⁺ T cells that were induced to undergo apoptosis outside the margin of the tumor sites in these latter groups of animals. While we have observed infiltration of tumor-reactive TGF- β -insensitive CD8⁺ T cells into the tumor parenchyma, we have also observed the occasional presence of other immune cells (CD4⁺, NK, B cells). In other treatment groups (those who received adoptive transfer of naïve CD8⁺ T cells or tumor-reactive control CD8⁺ T cells), these non-CD8⁺ T immune cells were not observed in the tumor parenchyma. It is unclear whether these non-CD8⁺ T cells infiltrated into the tumor parenchyma by a yet unknown mechanism or diffused into the tumor tissue alone with the tumor-reactive TGF- β -insensitive CD8⁺ T cells. Nevertheless, the presence of other immune cells in the tumor parenchyma suggests a possible interaction between tumor-reactive TGF- β -insensitive CD8⁺ T cells with other types of immune cells and warrants further investigation in the future.

In summary, adoptive transfer of tumor-reactive TGF- β -insensitive CD8⁺ T cells resulted in infiltration of these immune cells into the tumor parenchyma, secretion of relevant cytokines, and induction of apoptosis in tumor cells. These results support the concept that TGF- β is an important target in cancer immunotherapy.

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Blockade of transforming growth factor- β signaling in tumor-reactive CD8⁺ T cells activates the antitumor immune response cycle

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Abstract

Transforming growth factor- β (TGF- β) is a potent immunosuppressant. Overproduction of TGF- β by tumor cells leads to evasion of host immune surveillance and tumor progression. Results of our early studies showed that adoptive transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells into immunocompetent mice was able to eradicate lung metastasis of mouse prostate cancer. The present study was conducted with three objectives. (a) We tested if this technology could be applied to the treatment of solid xenograft tumors in allogeneic immunodeficient hosts. (b) We determined relevant variables in the tumor microenvironment with the treatment. (c) We tested if immune cells other than CD8⁺ T cells were required for the antitumor effect. Mouse prostate cancer cells, TRAMP-C2 of the C57BL/6 strain, grown in immunodeficient allogeneic hosts of BALB/c strain, were used as a xenograft model. Tumor-reactive CD8⁺ T cells from C57BL/6 mice were isolated, expanded *ex vivo*, and rendered insensitive to TGF- β by introducing a dominant-negative TGF- β type II receptor vector. Seven days

following s.c. injection of TRAMP-C2 cells (5×10^5) into the flank of male BALB/c-Rag1^{-/-} mice, tumor-reactive, TGF- β -insensitive CD8⁺ T cells (1.5×10^7) were transferred with and without the cotransfer of an equal number of CD8-depleted splenocytes from C57BL/6 donors. Naive CD8⁺ T cells or green fluorescent protein-empty vector-transfected tumor-reactive CD8⁺ T cells were transferred as controls. Forty days following the transfer, the average tumor weight in animals that received cotransfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells and CD8-depleted splenocytes was at least 50% less than that in animals of all other groups ($P < 0.05$). Tumors in animals of the former group showed a massive infiltration of CD8⁺ T cells. This was associated with secretion of relevant cytokines, decreased tumor proliferation, reduced angiogenesis, and increased tumor apoptosis. Based on these results, we postulated a concept of antitumor immune response cycle in tumor immunology. [Mol Cancer Ther 2006;5(7):1-11]

Introduction

Tumor immunology is characterized by an insufficient immune surveillance, as most tumors are able to evade the immune surveillance program of the host. Despite the ability of generating the reactivity of immune cells against tumor antigens, the immune surveillance program of the host can be overpowered by tumors with an eventual tumor progression (1). This is because tumor cells have acquired many mechanisms to evade the immune surveillance program of the host (2, 3). One of such possibilities has been the tumor-derived transforming growth factor- β (TGF- β), which is highly immunosuppressive (3–6). Most tumors secrete large amounts of TGF- β (7–9). TGF- β -producing tumor cells fail to elicit primary CTL responses despite retaining class I MHC expression molecules and tumor-specific antigens (4). Priming of T cells by dendritic cells or tumor cells can also be negatively influenced by TGF- β (10). Therefore, an ideal approach to activate the antitumor response will be to render the immune cells of the host insensitive to TGF- β .

In 2001, Gorelik and Flavell disrupted TGF- β signaling in CD4⁺ and CD8⁺ T cells through the transgenic expression of a truncated dominant-negative TGF- β type II receptor (T β RIIDN). Adoptive transfer of these T cells allowed the generation of an immune response capable of inhibiting metastasis in mice challenged with murine thymoma EL-4 and melanoma B16 cells (11). In 2002, we reported the inhibition of metastasis of mouse prostate cancer TRAMP-C2 and mouse melanoma B16 by transplanting TGF- β -insensitive bone marrow cells into mice (12, 13). In all studies described above, although antitumor response

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was encouraging, the nonspecific nature of the immune reaction led to the widespread inflammatory disease in the hosts (12). Most recently, we employed adoptive transfer of tumor-specific TGF- β -insensitive CD8⁺ T cells to tumor-bearing immunocompetent mice and were able to eradicate established lung metastasis of TRAMP-C2 tumors (14, 15). This approach showed no apparent development of the widespread inflammatory syndrome in the recipients and therefore offers a possibility for clinical application.

Although the above initial observations are encouraging, further characterization of this novel approach is necessary. (a) We would like to know if the system could be applied to the treatment of solid tumors. (b) We also would like to determine if this approach can be used to test antitumor efficacy in allogeneic hosts, so that we will be able to test clinical specimens in immunodeficient surrogate animals. (c) We would like to identify the major players in the current system of adoptive transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells for cancer therapy. In the present study, we employed the above approach to investigate the ability of tumor-reactive, TGF- β -insensitive CD8⁺ T cells on primary solid tumors using the allogeneic immunodeficient mice as a surrogate host. Here, we report solid tumor response, alteration of tumor microenvironment, and systemic and local cytokine response and postulate the concept of an antitumor immune response cycle.

Materials and Methods

Experimental Animal and Cell Lines

Male BALB/c-Rag1^{-/-} strain Rag1 mice 6 to 8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Northwestern University. TRAMP-C2 is an early-passage androgen-independent prostate cancer cell line derived from TRAMP mouse (C57BL/6 strain) that developed prostate cancer due to its prostate-specific expression of just SV40 T antigen that drives the prostate cancer development in that model (16). The mouse melanoma cell line B16-F10 was obtained from the American Type Culture Collection. Both cell lines were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA).

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Ex vivo Expansion of Tumor-Reactive, TGF- β -Insensitive CD8⁺ T Cells

Primed tumor-reactive CD8⁺ T cells were isolated from C57BL/6 mice that were vaccinated five times each with irradiated TRAMP-C2 cells (5×10^6 per mice per injection). The *ex vivo* culture was done as described previously (14). Tumor-reactive CD8⁺ T cells were rendered insensitive to TGF- β by infection with T β RIIDN-green fluorescent protein (GFP)-containing retrovirus as described previously (12, 13). Infection efficiency was assessed by GFP expres-

sion and flow cytometry and was always >90%. Naive spleen cells were isolated from the C57BL/6 mice and the depletion of CD8⁺ T cells was done by using MagCelect Magnet apparatus (R&D Systems, Minneapolis, MN) with a biotinylated antimouse CD8 α antibody and MagCelect streptavidin ferrofluid (R&D Systems) according to the manufacturer's protocol. *In vitro* cytotoxic assay was done by ⁵¹Cr release assay as described previously (13).

Challenge of the Mouse Prostate Cancer and Adoptive Transfer of CD8⁺ T Cells

BALB/c-Rag1^{-/-} mice received an injection in the right flank with 5×10^5 TRAMP-C2 cells. Seven days later, adoptive transfer with CD8⁺ T cells was done. Each group (5–12 mice per group) received i.p. transfer of one of the following six groups of CD8⁺ T cells (1.5×10^7) with or without the same amount of naive CD8-depleted splenocytes. In group 1 (12 mice) and group 2 (10 mice), tumor-reactive, TGF- β -insensitive CD8⁺ T cells were transferred with or without CD8-depleted splenocytes, respectively. Group 3 (10 mice) and group 4 (10 mice) received tumor-reactive, TGF- β -sensitive CD8⁺ T cells infected with or without the cotransfer of CD8-depleted splenocytes, respectively. Group 5 (10 mice) and group 6 (5 mice) received naive CD8⁺ T cells with or without cotransfer of CD8-depleted splenocytes, respectively. Tumor size was measured weekly. Forty days after the adoptive transfer, all mice were sacrificed and the tumors were isolated for evaluation of the volume, weight, and histologic characteristics. Tumor volumes were estimated using the formula: volume = $0.5 \times [(\text{length} + \text{width}) \times \text{length} \times \text{width}] \times 0.5236$.

Determination of Interleukin-2 and IFN- γ in Serum by ELISA

Blood was extracted from a central artery of mice from each treatment group. The supernatant was separated by allowing the whole blood to stand at room temperature for 2 hours. ELISA assays were carried out using the Quantikine mouse interleukin-2 (IL-2) and IFN- γ immunoassay kits (R&D Systems) according to the manufacturer's protocol. Expression of cytokines in tumor parenchyma was evaluated by an immunofluorescent approach as discussed below.

Pathologic Evaluation and Immunohistochemical Staining

After mice were euthanized, the tumor from each animal was excised, fixed in formalin, and embedded in paraffin. Sections (4 μ m) were obtained. Routine H&E staining was done on every fifth serial section. All H&E sections were evaluated by at least two independent investigators. CD31, Ki-67, and Bcl-2 were used for immunohistochemical staining in conjunction with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. After deparaffinization, quenching of endogenous peroxidase activity, and normal serum pre-blocking, the sections were incubated in either diluted mouse monoclonal antibody against CD31 (1:100; Upstate, Lake Placid, NY), Ki-67 (1:200; DAKO, Carpinteria, CA), or Bcl-2 (1:100; Upstate) for 2 hours at room temperature.

This was followed by incubation with biotinylated goat anti-mouse secondary antibody (1:200; Vector Laboratories) for 2 hours. Peroxidase substrate solution 3,3'-diaminobenzidine (DAKO) was used for direct staining. Harris hematoxylin solution was used for counterstaining.

Immunofluorescent Staining and Apoptosis Assay

Unstained paraffin-embedded serial sections of spleen and tumor were used for immunofluorescent staining to detect presence of transferred CD8⁺ T cells in spleen tissue (nuclear-CD8⁺-GFP protein triple staining) and tumor nodules [nuclear-CD8⁺-terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) triple staining]. Nuclear-IFN- γ /IL-2 double staining was also done on these sections to analyze secretion of cytokines in tumor parenchyma. The primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunofluorescent costaining was done by using the assay as described previously (12–14). The TUNEL apoptosis assay kit (Upstate) was used according to the manufacturer's protocol. Briefly, slides were treated with proteinase K for 30 minutes at 37°C and incubated with a terminal deoxynucleotidyl transferase end labeling cocktail (terminal deoxynucleotidyl transferase buffer, biotin-dUTP, and terminal deoxynucleotidyl transferase at a ratio of 90:5:5) for 120 minutes at 37°C. This was followed by overlaying an avidin-FITC (green) solution (50 μ L) and incubated in the dark for 60 minutes at 37°C. Slides were then incubated with 50 μ L blocking buffer at room temperature for 20 minutes followed by a rat monoclonal antibody for CD8 labeled with Texas red (2 μ g/mL; Santa Cruz Biotechnology) in the dark for 30 minutes at room temperature. All slides were stained with Vectashield mounting medium (blue; Vector Laboratories) for nuclear counterstaining. Slides were examined with a Nikon TE2000-U fluorescent microscope (Nikon Corp., Tokyo, Japan). Images were digitized by Photoshop 7.0. The intensity of the fluorescent signal was standardized by the standard fluorescent index (positive lymphocytes or signal/100 tumor cells/1,000 μ m²: –, <5; \pm , 6–10; +, 11–30; ++, 31–50; +++, 51–70; +++, >70).

Statistical Methods

ANOVA and multiple range tests were done to determine differences of means among different treatment groups. $P < 0.05$ was considered statistically significant. SPSS 10.0.7 software package (SPSS, Inc., Chicago, IL) was used for analysis.

Results

Reduced Tumor Burden in Allogeneic Immunodeficient Hosts

Q4 The specific tumor-killing ability of the tumor-reactive, TGF- β -insensitive CD8⁺ T cells was shown by the *in vitro* CTL assay (Fig. 1A). These cells showed a 5- and 25-fold greater tumor-killing activity than the TGF- β -sensitive counterparts and naive CD8⁺ T cells, respectively. Both TGF- β -sensitive and TGF- β -insensitive tumor-reactive CD8⁺ T cells showed a reduced tumor-killing activity

when incubated with an irrelevant cell line, mouse B16-F10 melanoma cells (Fig. 1B). In the group treated with cotransfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells and CD8-depleted splenocytes, 2 of 12 mice were free of tumor, and the tumor burden in the remaining 10 mice was 50% less than that of animals in other groups ($P < 0.05$; Fig. 1C and E). The average tumor volumes and tumor weights were not significantly different within the other five groups. In the group treated with the tumor-reactive, TGF- β -insensitive (T β RIIDN) CD8⁺ T cells with or without the cotransfer of CD8-depleted splenocytes (group 2), the tumor burden was not significantly different from that of wild-type tumor-reactive CD8⁺ T cells or naive groups with or without the cotransfer of CD8-depleted splenocytes (groups 3–6). Furthermore, tumor growth rate curves were generated and shown in Fig. 1F based on the tumor volume measurement weekly. The tumor growth rates correspond to the final tumor volumes (Fig. 1C and D) in each group. The tumor growth rate was significantly inhibited by treatment of TGF- β -insensitive (T β RIIDN) CD8⁺ T cells with the cotransfer of CD8-depleted splenocytes (group 1) when compared with other five groups. The data would suggest that the transfer of CD8-depleted splenocytes improves the efficacy of adoptive transfer with the modified CD8⁺ T cells.

Adoptively Transferred Tumor-Reactive, TGF- β -Insensitive CD8⁺ T Cells Persisted in the Spleen of the Host

Adoptively transferred CD8⁺ T cells were detected in the spleen of recipient animals (Fig. 2A and B), suggesting that these CD8⁺ T cells were able to persist in recipient hosts at least at the time of sacrifice, which was 40 days since the initial adoptive transfer. In contrast, in animals that received the wild-type tumor-reactive CD8⁺ T cells or naive CD8⁺ T cells with or without the cotransfer of CD8-depleted splenocytes (groups 2–6), only occasional CD8⁺ T cells were detected in the spleen (Fig. 2A and B). These results suggested that, in immunodeficient mice (Rag1^{-/-}), cotransfer of wild-type CD8⁺ T cells and CD8-depleted splenocytes was unable to manifest an engraftment of transferred cells in the recipients unless tumor-reactive, TGF- β -insensitive CD8⁺ T cells were cotransferred with the CD8-depleted splenocytes.

Drastic Histologic Changes in Tumor Tissues (H&E, Ki-67, Bcl-2, and CD31 Staining)

Three main histologic features in tumors of animals in group 1 differed from those of the other five groups (Fig. 3; Table 1). (a) The tumors in mice that received cotransfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells and CD8-depleted splenocytes (group 1) had heavy infiltration of lymphocytes into the tumor parenchyma. (b) There was a significant increase of spindle-shaped cells, suggesting degeneration of cancer cells. (c) There was a significantly less number of mitosis (0.5 versus 3–5 per $\times 400$ field). These findings are consistent with the immunohistochemical staining for Ki-67. As shown in Fig. 3, most tumor cells (>90%) in animals of groups 2 to 5 stained strongly with Ki-67 and Bcl-2. In sharp contrast, the degenerative tumor cells in mice of group 1 showed far less intensity and

4 TGF- β Signaling in Tumor-Reactive CD8⁺ T Cells

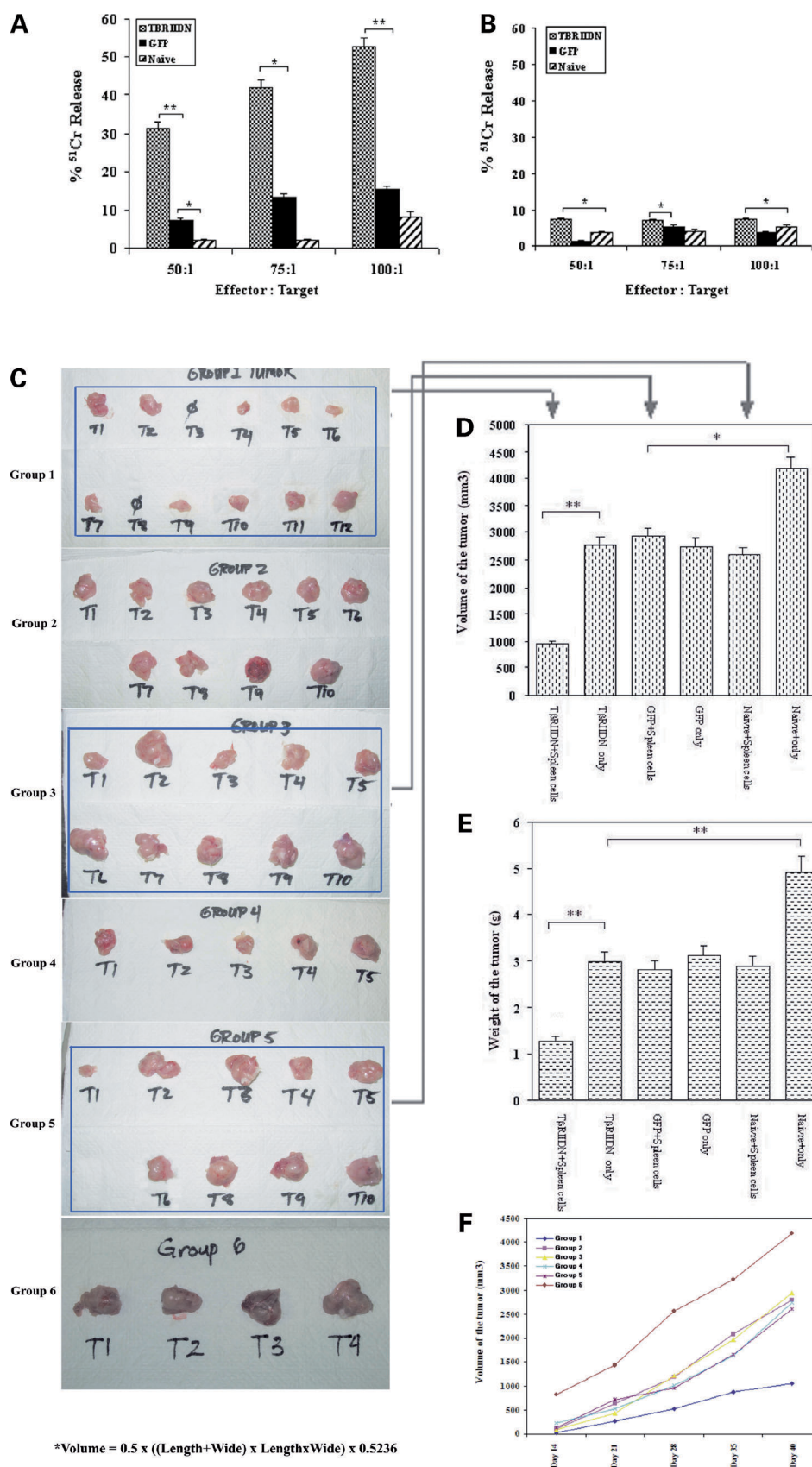
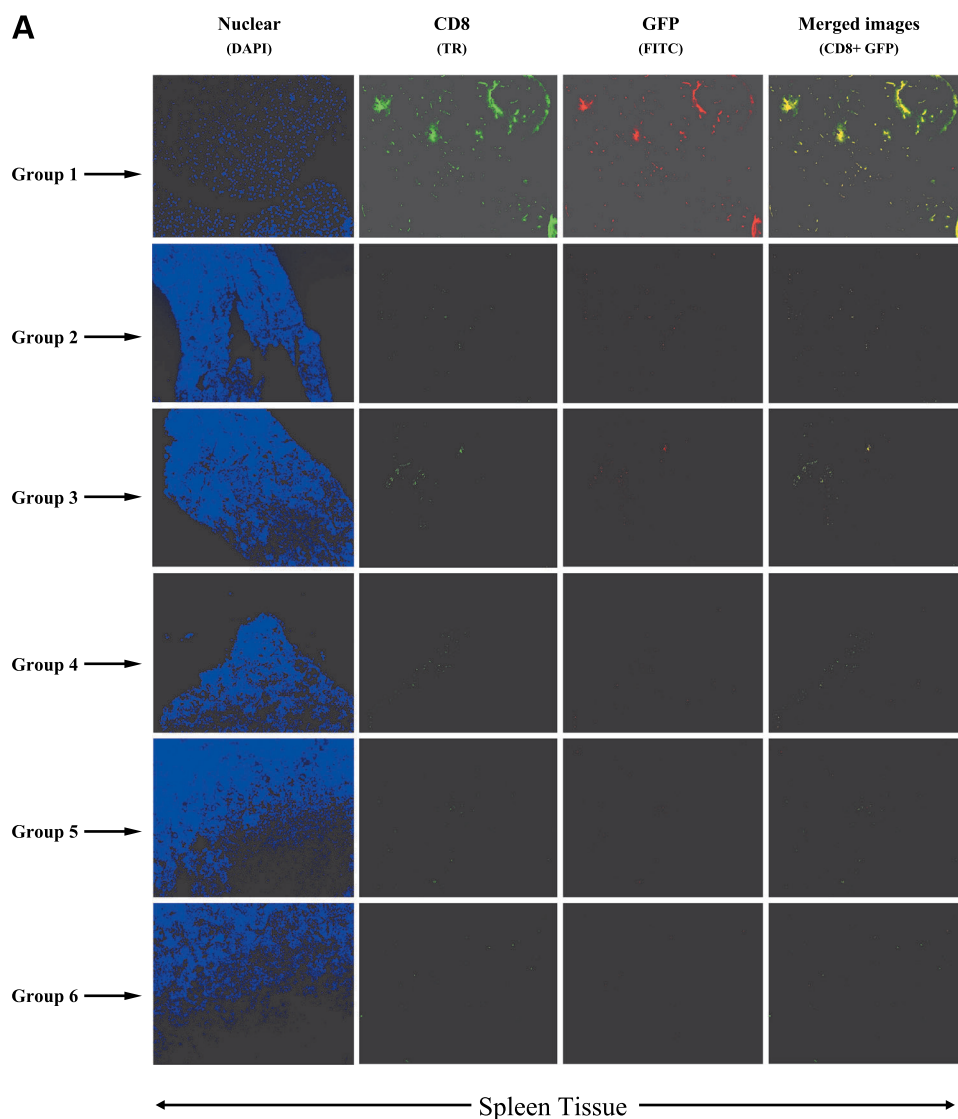


Figure 1. *In vivo* antitumor activity of tumor-reactive, TGF- β -insensitive CD8⁺ T cells. Three types of CD8⁺ T cells were used for *in vitro* chromium release assay: naive CD8⁺ T cells from untreated C57BL/6 mice (Naive), tumor-reactive control CD8⁺ T cells (GFP), and tumor-reactive, TGF- β -insensitive CD8⁺ T cells (T β RIIDN). **A**, TRAMP-C2 mouse prostate cancer cells were used as the targets. **B**, B16-F10 mouse melanoma cells were used as targets. **Columns**, average observation obtained from eight wells; **bars**, SD. $P < 0.05$, T β RIIDN CD8⁺ T cells versus the GFP and naive CD8⁺ T cells. Six groups of CD8⁺ T cells (1.5×10^7) with or without the same amount of naive CD8⁺ T cell-depleted spleen cells were used. In groups 1 and 2, tumor-reactive, TGF- β -insensitive CD8⁺ T cells infected with the T β RIIDN-GFP viral particles were transferred with or without CD8⁺ depletion spleen cells, respectively. In groups 3 and 4, tumor-reactive, TGF- β -sensitive CD8⁺ T cells were infected with the GFP viral particles only with or without the cotransfer of CD8⁺-depleted spleen cells, respectively. In groups 5 and 6, naive CD8⁺ T cells were adoptively transferred with or without cotransfer of CD8⁺-depleted spleen cells, respectively. TRAMP-C2 cells (5×10^5) were challenged to recipient mice. At 7 d following tumor challenge, the different subtypes of CD8⁺ T cells were transferred through i.p. injection with or without cotransfer of spleen cells. Animals were sacrificed at 40 d following the adoptive transfer. **C**, representative gross features of prostate cancer samples from tumor-bearing mice at 40 d following adoptive transfer. **D**, weight of the tumor of each group. T β RIIDN-treated mice completely abolished tumors in 2 mice, with the remaining 10 bearing the smallest and lightest tumor burden. $P < 0.05$, T β RIIDN + spleen group versus the GFP group, naive group, and T β RIIDN only group in both weight and volume. Furthermore, tumor growth rate was inhibited significantly in T β RIIDN-treated mice with cotransfer of CD8-depleted splenocytes. **F**, curve was generated based on the tumor volume measurement weekly. The tumor growth rate is corresponding to the final tumor volumes (**C** and **D**) in each group.

Figure 2. Infiltration of lymphocytes into the spleen tissue. **A**, in contrast to GFP-infected and naive CD8⁺ T cells, only T β RIIDN + spleen group CD8⁺ T cells effectively infiltrated into the spleen tissue. **B**, CD8⁺ T-cell infiltration in spleen tissue was described as a standard fluorescent index (positive signal/1,000 spleen cells/1,000 μ m²: -, <5; \pm , 6-10; +, 11-30; ++, 31-50; +++, 51-70; +++, >70).



B

Infiltration of CD8⁺ T cells into spleen tissue:

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
CD8 ⁺ T cells	++++	+	\pm	\pm	\pm	\pm
Infiltration						

• For spleen tissue: positive signal/1000 spleen cells/1000 μ m²: -, <5; \pm , 6-10; +, 11-30; ++, 31-50; +++, 51-70; +++, >70

density of the same markers. For CD31, as illustrated in Fig. 3, tumors from animals in groups 2 to 5 contained significantly more CD31⁺ cells than those from animals in group 1. Quantitative analysis revealed that the microvessel densities (CD31⁺) in tumors of animals in groups 1 to 6 were $26 \pm 8/\text{mm}^2$, $177 \pm 37/\text{mm}^2$, $154 \pm 45/\text{mm}^2$, $196 \pm 22/\text{mm}^2$, and $164 \pm 41/\text{mm}^2$, and $121 \pm 28/\text{mm}^2$, respectively (Table 1). These observations are consistent with the results of the TUNEL assay (Fig. 3). Results of immunohistochemical staining for Bcl-2 showed scant

staining in tumors of animals in group 1 in comparison with that in tumors of groups 2 to 5, which stained strongly for Bcl-2. A quantitative summary of expression of H&E, Ki-67, CD31, and Bcl-2 is listed in Table 1.

Infiltration of CD8⁺ T Cells Into the Tumor Parenchyma and Induced Tumor Cells Apoptosis

The most prominent histologic feature of the tumor tissue in this study was the evidence of infiltration of many tumor-reactive, TGF- β -insensitive (GFP⁺) CD8⁺ T cells with concomitant apoptosis in tumor tissues in animals of

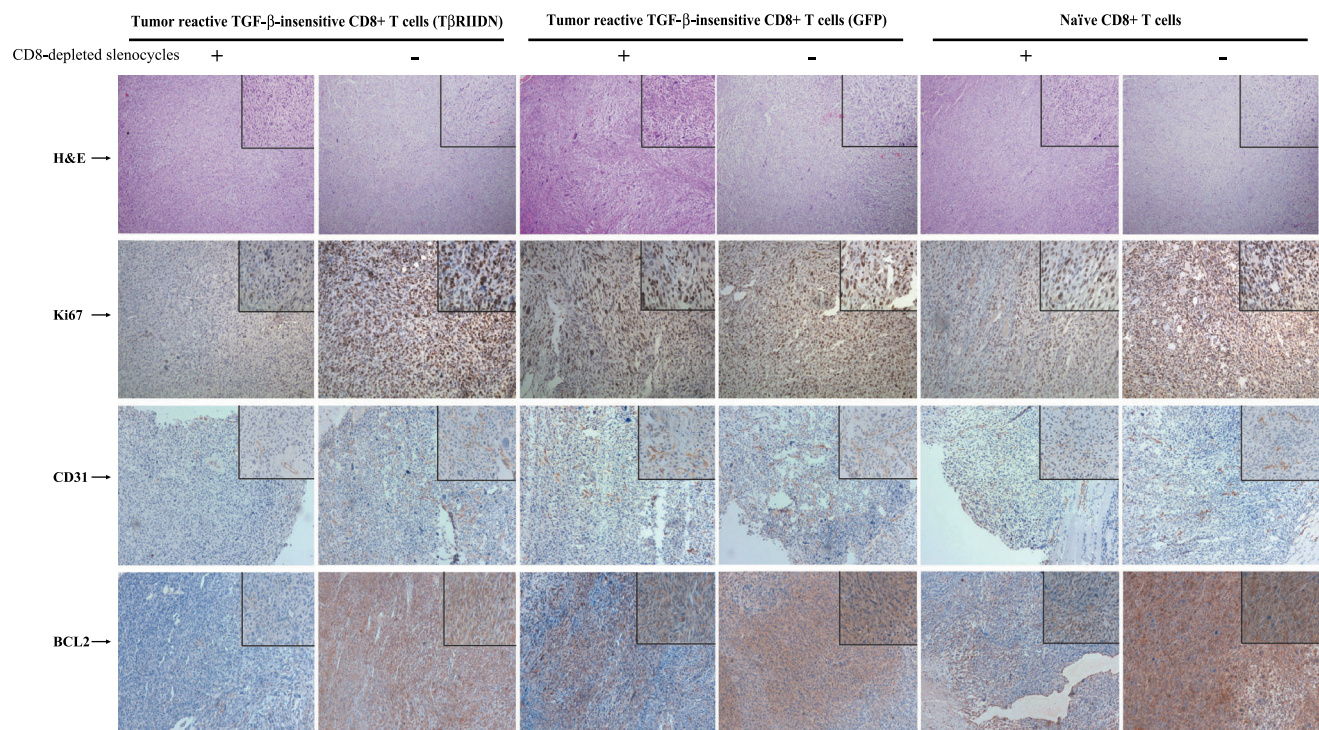


Figure 3. Histologic evaluation of the tumor. H&E staining in the tumors showed that mice that received adoptively transferred tumor-reactive, TGF-β-insensitive CD8⁺ T cells and spleen cells (TβRIIDN + spleen) had heavy lymphocytic infiltrates into tumor parenchyma compared with the other five groups. This group also showed a significant increase in cell spindling and degenerative appearance. There were significantly lower measurable mitoses (0.5 in average per ×400 field) than the other groups (3–5 per ×400 field). This finding corresponded to the immunohistochemical staining for Ki-67. Most cells (>90%) in tumors of GFP, naive groups, and TβRIIDN only treatment group stained strongly for Ki-67 and Bcl-2. The intensity and density of the staining was much weaker in the degenerating tumors in mice that received tumor-reactive, TGF-β-insensitive CD8⁺ T and spleen cells. CD31 staining in tumors from GFP, naive, and TβRIIDN only treatment groups was significantly more intense than in tumors from TβRIIDN + spleen group.

group 1 (Fig. 4A and B). The degree of infiltration by different types of lymphocytes was evaluated by the standard fluorescent index, which corresponded to the fluorescent intensity criterion (positive lymphocytes/100 tumor cells/1,000 μm²: –, <5; ±, 6–10; +, 11–30; ++, 31–50;

+++, 51–70; +++++, >70; Fig. 4A). Almost all of these infiltrated CD8⁺ T cells were GFP⁺, consistent with the knowledge that all these CD8⁺ T cells were adoptively transferred. Results of the TUNEL assay revealed that apoptosis in tumor cells was detected only in animals of

Table 1. Characteristics of histologic finding

	Tumor-reactive, TGF-β-insensitive CD8 ⁺ T cells (TβRIIDN)		Tumor-reactive, TGF-β-sensitive CD8 ⁺ T cells (GFP)		Naive CD8 ⁺ T cells	
CD8-depleted splenocytes*	+	–	+	–	+	–
Mitosis [†]	0.5 ± 0.12	3.5 ± 0.67	4.1 ± 1.41	3.5 ± 1.21	4.6 ± 0.87	5.1 ± 2.1
Degeneration of cancer cells	Yes	No	No	No	No	No
Infiltration of lymphocytes	Yes	No	No	No	No	No
Spindly change of cancer cells	Yes	No	No	No	No	No
Ki-67 [‡]	+	+++	+++	+++	+++	+++
CD31§	26 ± 8	177 ± 37	154 ± 45	196 ± 22	164 ± 41	121 ± 28
Bcl-2 [‡]	+	+++	+++	+++	+++	+++

NOTE: Quantitative analysis revealed that the microvessel densities in tumors of TβRIIDN + spleen, TβRIIDN only, GFP + spleen, GFP only, naive + spleen, naive only were 26 ± 8/mm², 177 ± 37/mm², 154 ± 45/mm², 196 ± 22/mm², 164 ± 41/mm², and 121 ± 28/mm² respectively. In addition, immunohistochemical staining for Bcl-2 showed the least intense staining in TβRIIDN + spleen group. The characteristics of H&E and quantitative expression of Ki-67, Bcl-2, and CD31 of different groups are listed.

*Large-sized window is with a magnification of ×100; small-sized window is with a magnification of ×400.

[†]Mitosis was expressed by the number of mitosis for each ×400 field.

[‡]Evaluation of the staining of Ki-67 and Bcl-2: ±, < 5%; +, 5–30%; ++, 30–50%; +++, > 50%.

§Quantitative analysis of CD31 was evaluated by microvessel density per mm².

group 1. Tumors in the animals of the other five groups showed little or no infiltration of CD8⁺ T cells and showed no evidence of apoptosis in tumor cells.

Up-Regulation of Systemic and Local Levels of IFN- γ and IL-2

An increase in serum level of IL-2 and IFN- γ (Fig. 5A and B) was observed in animals cotransferred with tumor-reactive, TGF- β -insensitive CD8⁺ T cells and CD8-

depleted splenocytes (group 1), suggesting the presence of activated immune cells. Increased levels of IFN- γ and IL-2 were also noted by immunofluorescent analysis in the tumor parenchyma, which correlated with serum levels (Fig. 5C). IFN- γ and IL-2 was localized around CD8⁺ cells, which imply that these cytokines were produced by these immune cells. In comparison, IFN- γ and IL-2 expression in tumors of animals in other five

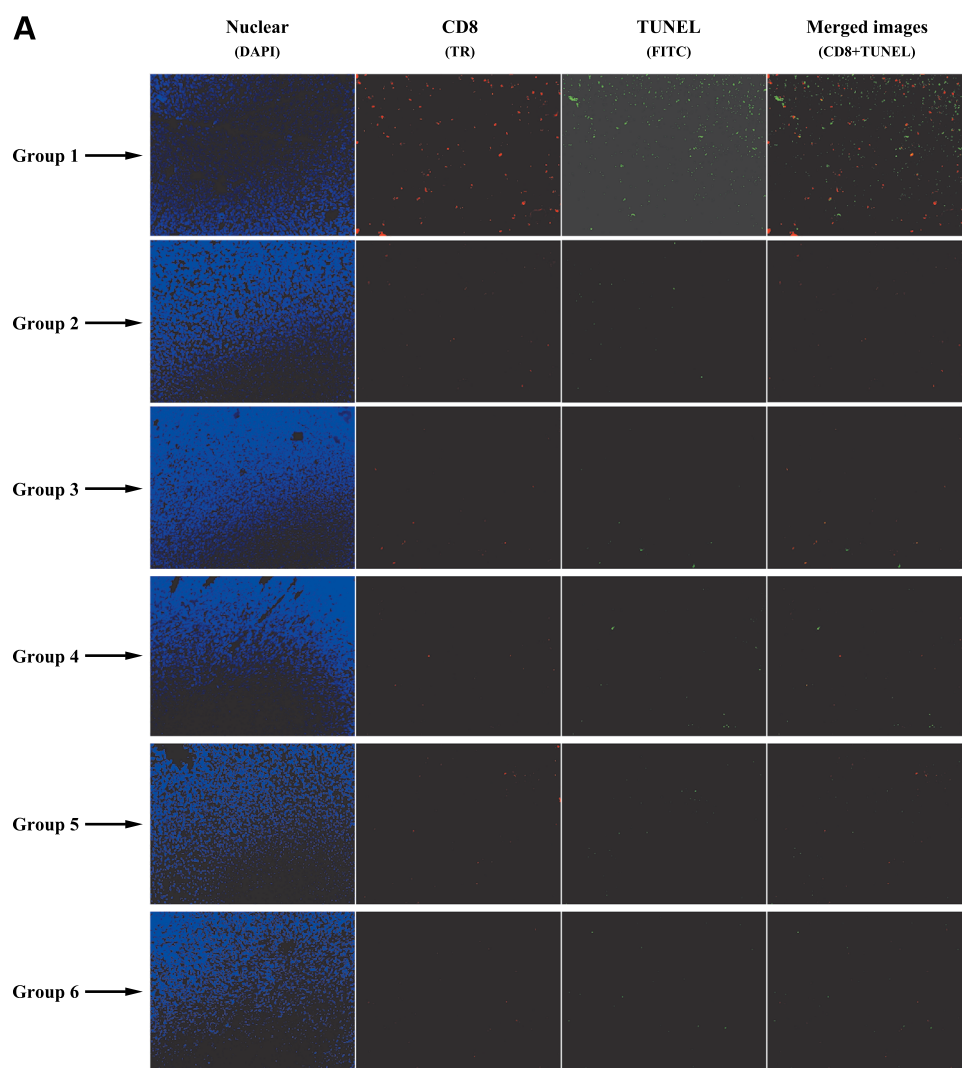
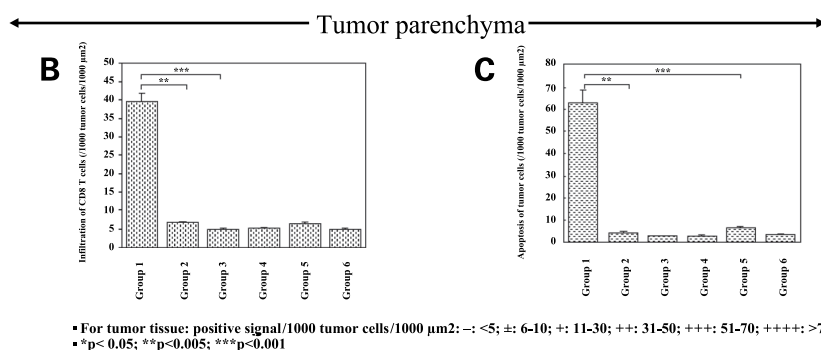


Figure 4. Infiltration of lymphocytes into tumor parenchyma and apoptosis of tumor cells. **A**, representative tissue sections were simultaneously stained for cell nucleus (blue), CD8⁺ T cells (red), and apoptosis (green). The tumor site was identified by the nuclear staining (blue). CD8⁺ T cells were localized in the tumor parenchyma that also stained for tumor apoptosis (green) in the T β RIIDN + spleen group. The majority of the apoptotic cells were tumor cells (green) and not CD8⁺ T cells (yellow). Magnification, $\times 400$. In contrast, tumors in animals that received CD8⁺ cells that were either naive, GFP alone, or treated with T β RIIDN only did not exhibit significant infiltration of CD8⁺ T cells or tumor cell apoptosis within the tumor parenchyma. In contrast to GFP-infected and naive CD8⁺ T cells, only T β RIIDN + spleen group CD8⁺ T cells effectively infiltrated into both tumor parenchyma. **B**, quantitative analysis CD8⁺ T cell infiltration. **C**, TUNEL assay of tumor tissue.



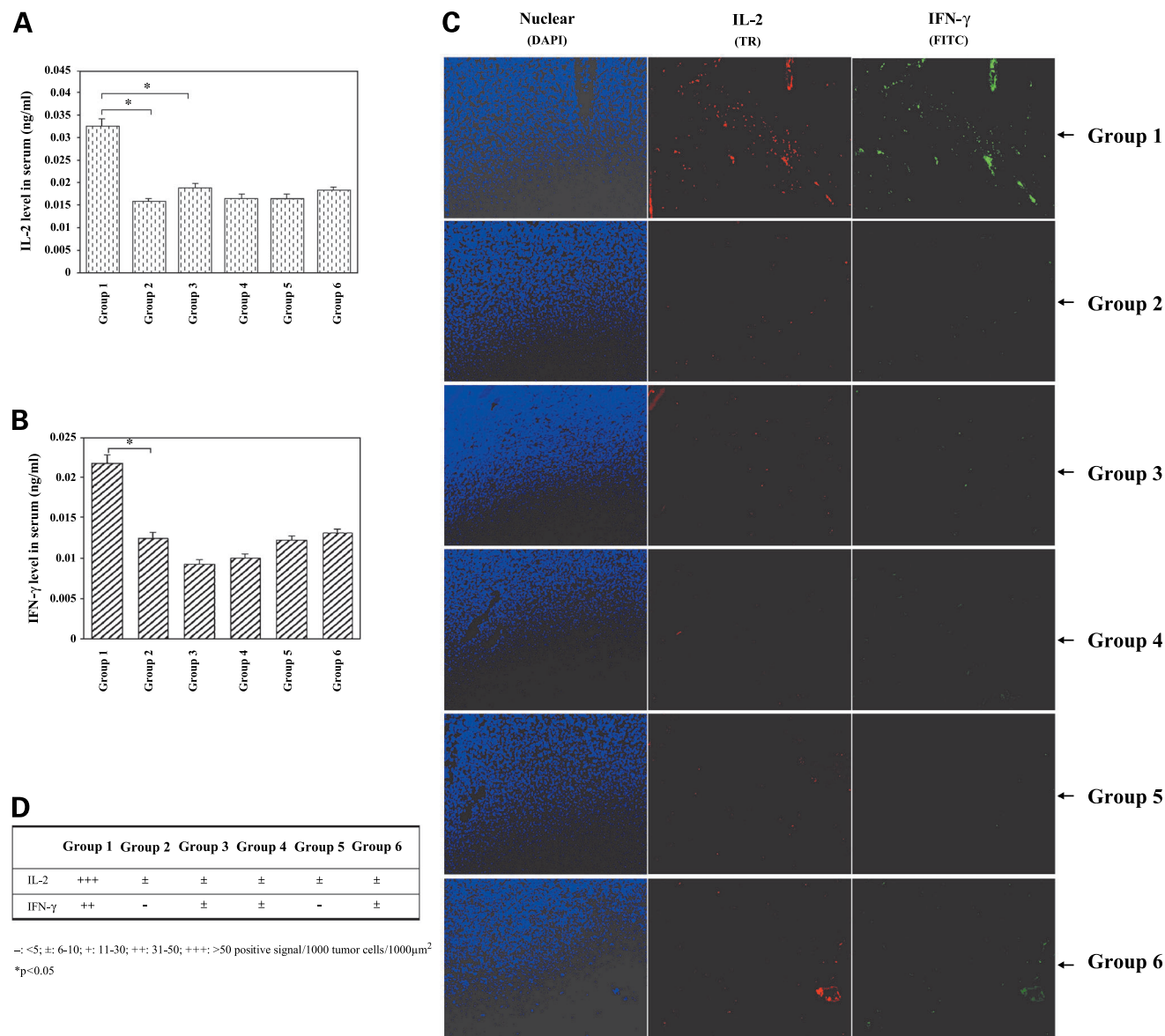


Figure 5. Secretion of cytokines in tumor of different groups. In animals that received adoptive transfer with or without cotransfer of spleen cells, there was a baseline level of IL-2 and IFN-γ. Increased serum levels of IL-2 (**A**) and IFN-γ (**B**) were observed in mice adoptively transferred with tumor-reactive CD8⁺ T cells that were rendered insensitive to TGF-β (TβRIIDN + spleen cells group) compared with mice in the control groups [naive CD8⁺ T cells or tumor-reactive control CD8⁺ T cells (GFP group)]. IFN-γ and IL-2 expression from the GFP group, naive CD8⁺ T cell group, and TβRIIDN only group was significantly lower than the treatment group. Local levels of IFN-γ and IL-2 were evaluated by immunofluorescent staining. **C**, high levels of IFN-γ and IL-2 staining were observed in tumor tissue in the TβRIIDN + spleen group compared with all other groups. **D**, cytokine expression was described as a standard fluorescent index (positive signal/1,000 tumor cells/1,000 μm²: —, <5; ±, 6–10; +, 11–30; ++, 31–50; +++, 51–70; +++++, >70).

groups (groups 2–6) was negligible. A summary of cytokine expression was described as a standard fluorescent index listed in Fig. 5D.

Discussion

Results of the present study have provided three important pieces of information. (a) Our results have shown that it is feasible to use immunodeficient allogeneic mice as sur-

rogate hosts for the treatment of xenograft tumors by adoptive transfer of tumor-reactive, TGF-β-insensitive autologous CD8⁺ T cells. (b) We have indicated that the present treatment protocol created a tumor microenvironment that favorably eliminated the s.c. solid tumors. (c) We showed that the adoptively transferred tumor-reactive, TGF-β-insensitive CD8⁺ T cells alone were insufficient for an antitumor response unless they are support by other immune cells.

By using an allogeneic system, it is necessary for us to determine if graft-versus-host disease develops. Based on our results, it does not seem that within the timeline of this study there was any evidence of graft-versus-host disease. In the present study, the cotransferred CD8-depleted splenocytes seemed necessary to assist the tumor-reactive, TGF- β -insensitive CD8⁺ T cells to acquire the antitumor effector function. The cotransfer of CD8-depleted splenocytes was important, as CD8⁺ T cells are cytotoxic effector cells, which possess the ability to mediate apoptosis of target cells in the host. Had we cotransferred splenocytes that contained CD8⁺ T cells, these CD8⁺ T cells, on activation, would mediate apoptosis of the nontumor cells of the hosts, leading to autoimmune disease. Such examples are abundant in the literature. Our own experience has indicated that transfer of non-tumor-specific CD8⁺ T cells to recipients will lead to widespread inflammatory disease (12, 13). In the present study, the situation is more critical than the syngeneic systems, as the recipient is allogeneic to the transferred CD8⁺ T cells. Therefore, it is important that when we cotransfer splenocytes they must be CD8 depleted. Similarly, transfer of naive splenocytes will not be feasible, as they include CD8⁺ T cells.

In the normal prostate, TGF- β 1 signaling inhibits cell growth and induces apoptosis in epithelial cells (17, 18) and thus serves as a tumor suppressor. In prostate cancer, TGF- β promotes progression of advanced tumors through several paracrine and autocrine mechanisms (19). TGF- β secreted by tumor cells can facilitate tumor progression through stimulating proteins, such as vascular endothelial growth factor. TGF- β also induces expression of platelet-derived growth factor, connective tissue growth factor, and matrix metalloproteinases, all of which create conditions favorable for tumor invasion and angiogenesis (20). Further, TGF- β , being a potent immune suppressor, inhibits the immune system and facilitates tumor progression (4, 5, 21). The crucial role of TGF- β in maintaining immune system homeostasis is highlighted by the multifocal inflammatory disease that results from the genetic disruption of the TGF- β 1 allele in transgenic mice (22, 23). Conditional elimination of TGF- β signaling in T cells (24) or in bone marrow cells (25) results in a widespread inflammatory response.

In the present study, tumor-reactive, TGF- β -insensitive CD8⁺ T cells mediated complete regression of established solid tumors in 2 of 12 (16.7%) mice and inhibited >50% of the tumor burden in the remaining animals. This effect may be due to a reversal of the tumor-promoting microenvironment, which warrants further discussion. (a) Although tumor-derived TGF- β suppresses a variety of immune cells, we found that suppression of CTLs by TGF- β was the most critical (20). There was an 8-fold increase in tumor-reactive, TGF- β -insensitive CD8⁺ T cells that migrated into spleen tissue and the tumor parenchyma compared with those in control groups. This phenomenon was confirmed, in the present study, by the histologic analysis of spleen and tumor specimens. (b) The transferred tumor-reactive, TGF- β -insensitive CD8⁺ T cells induced systemic expression of

IL-2 and IFN- γ . It is likely that the up-regulation of these cytokines significantly enhanced the tumor-killing ability. (c) The present treatment protocol resulted in an inhibition of tumor cell proliferation as indicated by a decrease in Ki-67 staining intensity. This observation is consistent with the study of lung metastasis in which proliferating cell nuclear antigen expression was inhibited by the infiltration of tumor-reactive, TGF- β -insensitive CD8⁺ T cells. (d) The present result indicated that TGF- β -insensitive CD8⁺ T cells played a negative role in tumor angiogenesis (14). Angiogenesis is an important prognostic factor in cancer survival (26, 27). An increase in small vessels, as assessed by CD31 staining, correlated with lymph node involvement and was an independent predictor of survival in cancer patients. Various studies have highlighted the importance of CD8⁺ T cell, IL-2, and IFN- γ expression in inhibiting metastasis through blocking tumor angiogenesis (28–33). Our results showed that tumors in mice cotransferred with tumor-reactive, TGF- β -insensitive CD8⁺ T cells and CD8-depleted splenocytes were poorly vascularized. (e) Adoptive transfer of tumor-reactive wild-type CD8⁺ T cells could not effectively inhibit tumor growth regardless whether they were cotransferred with CD8-depleted splenocytes. Because these CD8⁺ T cells are the same as the conventional tumor-reactive CD8⁺ T cells (34, 35), this observation suggests that, in the face of high levels of tumor-derived TGF- β , adoptive transfer of conventional tumor-reactive wild-type CD8⁺ T cells would have limited antitumor efficacy. (f) Tumors in mice cotransferred with tumor-reactive, TGF- β -insensitive CD8⁺ T cells and CD8-depleted splenocytes showed markedly increased apoptosis, which coincided with an inhibition of Bcl-2 expression. Inhibitors of Bcl-2, such as ABT-737, can induce apoptosis in cancer cells and are potential agents in anticancer therapeutics (36–39). The expression of Bcl-2 was inhibited when a large number of tumor-reactive, TGF- β -insensitive CD8⁺ T cells infiltrated into the tumor parenchyma. In addition, cytokines, such as IL-2 and IFN- γ , may have played a coordinated role in the observed increase in tumor apoptosis. The regression of tumor cells, the presence of spindle-shaped cancer cells, the reduced mitotic figures, and the decreased expression of Bcl-2 may all contribute toward changes in the tumor microenvironment dictated by the presence of tumor-reactive, TGF- β -insensitive CD8⁺ T cells.

Tumor-reactive, TGF- β -insensitive CD8⁺ T cells showed a strong tumor-killing ability *in vitro*. Although CD8⁺ T cells are the cytotoxic effectors, CD4⁺ T cells are likely required to facilitate the effector function of CD8⁺ cells. Furthermore, CD4⁺ T cells can mediate CD8-independent antitumor function and memory (11, 40–42). Our *in vivo* results are consistent with these reports. Although we have not delineated the subtype of the immune cells in the spleen, the cotransferred CD8-depleted splenocytes included CD4⁺ cells, macrophages, and dendritic cells. This supports at least two possibilities. (a) Cotransfer of CD8-depleted splenocytes was required for the prolonged survival of CD8⁺ T cells. These splenocytes may contain antigen

presentation function to CD8⁺ T cells. Based on our observation, TGF- β -insensitive CD8⁺ T cells (T β RIIDN only group) alone could not be maintained unless CD8-depleted splenocytes were cotransferred. (b) Only with the cotransfer of CD8-depleted splenocytes could the CD8⁺ T cells reach the tumor parenchyma. This is likely due to the helper function of CD4⁺ T cells, which primed CD8⁺ T cells to acquire the antitumor effector function. Therefore, blockade of TGF- β signaling in tumor-reactive CD8⁺ T cells provides an effective antitumor function, which should be translated to the treatment of clinical cancer cases.

Based on the above results, we postulate the concept of an antitumor immune response cycle (Fig. 6). This antitumor immune response cycle represents a new paradigm in antitumor immunology and contains three major components: (a) tumor-reactive, TGF- β -insensitive CD8⁺ T cells; (b) the autologous tumor; and (c) the immune system of the host (Fig. 6). This concept will be briefly discussed below. (a) Results of the present study have indicated that tumor-reactive, TGF- β -insensitive CD8⁺ T cells are necessary for an effective antitumor immune response, as they are the only immune cells that are able to infiltrate into the tumor parenchyma and mediate tumor apoptosis. (b) Results of our past studies have indicated that the tumor itself is an important participant of this antitumor immune response cycle. The importance of autologous tumor has been shown by our earlier study (14, 15), which showed that in tumor-free hosts the tumor-reactive, TGF- β -insensitive CD8⁺ T cells were unable to persist in the spleen. (c) As shown by the present results, the presence of CD8-depleted immune cells is also necessary to manifest an effective antitumor immune response. This statement is based on the observation in

that, when tumor-reactive, TGF- β -insensitive CD8⁺ T cells were transferred alone, they were insufficient in the growth of the established s.c. TRAMP-C2 tumor. The system requires the cotransfer of CD8-depleted splenocytes in order for the transferred tumor-reactive, TGF- β -insensitive CD8⁺ T cells to mount an antitumor function (Fig. 6A). The immune response cycle can only be activated by tumor-reactive, TGF- β -insensitive CD8⁺ T cells infiltrating into tumor parenchyma and inducing apoptosis of tumor cells (Fig. 6B).

Up to this point, we have established three salient aspects of this new system. (a) The aspect was to show that adoptive transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells was able to eradicate established autologous tumors (14). (b) We showed that infiltration of transferred tumor-reactive, TGF- β -insensitive CD8⁺ T cells into the tumor parenchyma and to mediate tumor cell apoptosis was an important event in this system (15). (c) In this article, we report that the mere transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells was insufficient to mediate an antitumor response. Cotransfer of CD8-depleted splenocytes was necessary for the antitumor function of the transferred CD8⁺ T cells. In fact, the present observation was critical in that it allowed us to postulate the "antitumor immune response cycle." (d) We also obtain critical information from the present study that it is feasible for us to use allogeneic host for syngeneic tumor treatment with transfer of syngeneic immune cells. Such information will be missed if the present study was not conducted.

In an immunocompetent host, because the wild-type CD8⁺ T cells are unable to play any role in the antitumor immune response, they are not considered a part of this antitumor immune response cycle (Fig. 6A). The sequence of events of this antitumor immune response cycle starts with the infiltration of the tumor-reactive, TGF- β -insensitive CD8⁺ T cells into the tumor parenchyma to mediate tumor apoptosis and to release tumor-associated antigens into the circulation, thus allowing a continuous exposure of these antigens to the immune system of the host. It is likely that antigen-presenting cells, which are included in the CD8-depleted splenocytes, are important players in this process. Because antigen-presenting cells and CD4⁺ helper T cells are wild-type, they are unable to infiltrate into the tumor parenchyma. Their action must take place outside the tumor parenchyma. Therefore, the activation and priming of transferred tumor-reactive, TGF- β -insensitive CD8⁺ T cells must also take place outside of the tumor parenchyma. The primed CD8⁺ T cells, because they have been rendered TGF- β insensitive, are able to infiltrate into the tumor parenchyma (Fig. 6B). Such an antitumor immune response cycle will remain active until all tumor cells are eliminated.

In summary, results of the present study have provided three pieces of novel concept information. (a) Our results have shown that it is feasible to use immunodeficient mice as surrogate hosts for the treatment of solid cancer xenograft tumors with adoptive transfer of tumor-reactive, TGF- β -insensitive autologous CD8⁺ T cells. (b) There are

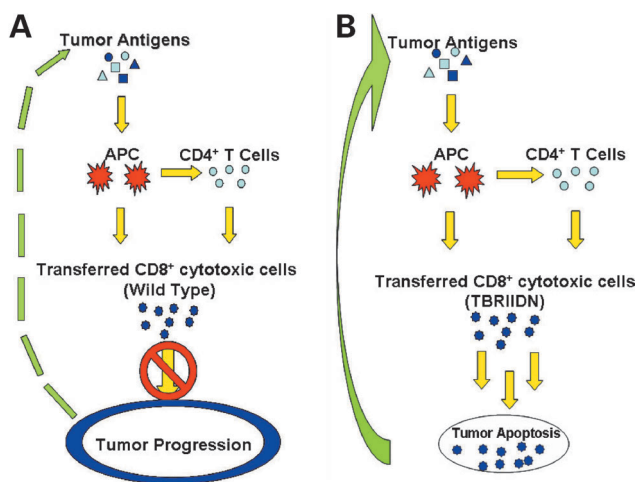


Figure 6. Adoptive transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells activates an otherwise incapacitated antitumor immune response cycle in tumor immunology. **A**, wild-type CD8⁺ T cells cannot infiltrate into the tumor. The antitumor immune response cycle ceases to function leading to tumor progression. **B**, CD8⁺ T cells are tumor specific and TGF- β insensitive, which can infiltrate into tumor parenchyma, and the antitumor immune response cycle is maintained, leading to tumor regression.

changes in the tumor microenvironment secondary to adoptive transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells as shown by changes in tumor histology, cytokine secretion, tumor cell proliferation, angiogenesis, and apoptosis. (c) We proposed a concept of antitumor immune response cycle, which consists of tumor-reactive, TGF- β -insensitive CD8⁺ T cells, the autologous tumor, and the immune system of the host. In an immunocompetent tumor-bearing host, by virtue of adoptive transfer of tumor-reactive, TGF- β -insensitive CD8⁺ T cells, the anti-tumor immune response cycle will be activated. This novel concept, although overly simplified, may lead to the development of effective antitumor therapeutic strategies in the near future.

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